University of Alberta

Development of Molecular and Microarray Techniques for the Detection of Viable *E. coli* O157:H7, *Vibrio cholerae* O1 and *Salmonella* Typhi

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Medical Sciences-Public Health Sciences

Edmonton, Alberta

Fall 2004

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ISBN: 0-612-95820-5
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ISBN: 0-612-95820-5

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Acknowledgements

For believing in my research proposal and providing financial support, I would like to thank the University of Alberta and the Province of Alberta for graduate student scholarships.

For providing research funding, such that I need not have worried about the cost of lab supplies, equipment and those many trips to Biochemistry stores, I would like to thank the Natural Sciences and Engineering Research Council of Canada (NSERC) and Alberta Health and Wellness.

For investing so much time and money in my graduate education, I would like to thank the University of Alberta, the Department of Public Health and especially the Environmental Health Sciences Group.

For allowing me to be her first graduate student, believing in my ability, and her encouragement to do my best, I would like to thank my supervisor, Dr. Xing Fang Li.

For his technical support, help with my experiments and most of all friendship, I would like to Dr. Zhilong Gong.

For their love, guidance, and support, I would like to thank my parents. For instilling in me a strong work ethic and a fun loving attitude, I would like to thank my father,

Raymond. For instilling in me strength, determination and independence, I would like to thank my mother, Margaret.

For those beautiful sunny days spent on their deck and a well stocked refrigerator, I would like to thank my sister Denise and my honorary brother Ryan.

For countless hours of entertainment, stupidity and hilarity, I would like to thank my pets: Boomer, Java, Cash, Dunkin' and Mikey. We miss you Boomer.

For your crisp, refreshing and soothing beverages, I would like to thank Mr. Big Rock and Mr. E.H. Rickards and my new favorite Mr. Sleeman.

Last but most importantly, I would like to thank my honey, Mike. Without your love, encouragement, support and patience, this journey would have been far more difficult. I dedicate this thesis to you.

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List of Abbreviations

A	Adenine
A/E	Attaching and effacing lesions
BLAST	Basic local alignment search tool
C	Cytosine
cDNA	Complementary DNA
CT	Cholera toxin
ddNTP	2',3'-dideoxynucleotide triphosphate
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
DTT	Dithiothreitol
G	Guanine
HC	Hemorrhagic colitis
HPLC	High performance liquid chromatography
HUS	Hemorrhagic uremic syndrome
LB	Liquid broth
LD_{50}	Lethal dose in 50% of the mice studied
LEE	Locus of enterocyte effacement
LPS	Lipopolysaccharide
LSW	Low salt wash
MBs	Molecular beacons
MPCR	Multiplex polymerase chain reaction
mRNA	Messenger RNA
MUG	4-Methylumbelliferyl β-D-glucoronide
MU	4-Methylumbelliferon
OD_{615}	Optical density at 615 nm
PBS	Phosphate buffer solution
PCR	Polymerase chain reaction
PS	Polysaccharide
RFLP	Restriction fragment linked polymorphism
RNA	Ribonucleic acid

rRNA	Ribosomal RNA
RT	Reverse transcriptase
RT-MPCR	Reverse transcriptase multiplex polymerase chain reaction
RT-PCR	Reverse transcriptase polymerase chain reaction
SBR	Signal-to-background ratio
SMAC	Sorbitol MacConkey agar
SNPs	Single nucleotide polymorphisms
Stx 1	Shiga toxin 1
Stx 2	Shiga toxin 2
T	Thymidine
TCBS	Thiosulphate citrate bile salts sucrose media
tRNA	Transfer RNA
UV	Ultraviolet
VBNC	Viable non-culturable cells
WHO	World Health Organization

Chapter 1: Introduction

1.1 Waterborne Outbreaks

Water is a necessity of life however, it is also a significant source of human disease and death [1]. Throughout history, deadly outbreaks of typhoid fever, cholera and dysentery were caused by contaminated drinking water. The link between human disease and water has not always been understood. During the 1800s, in London, UK, it was hypothesised that cholera was spread by "miasma" (a fog) originating from the Thames River [2]. In 1854, an English physician named John Snow demonstrated that cholera was actually transmitted through contaminated drinking water [3]. This link is even more impressive because it was made before the existence of bacteria was known [4]. In 1883, Robert Koch, a German microbiologist, identified *V. cholerae* bacteria in drinking water as the source of a cholera outbreak in Germany [5]. He also demonstrated the efficiency of water treatment in the prevention of cholera. Following a retrospective epidemiological study, he concluded that residents of Hamburg, Germany, suffered a higher mortality rate due to cholera than residents of Altona who received water from the same source [5]. In Altona, the only difference in the drinking water was that it had been treated by sand filtration prior to consumption. As a result of this ground-breaking discovery, major cities in Europe, the United Kingdom and North America began to treat their drinking water through filtration and chlorination [5].

In developed countries today, waterborne diseases are rare, but providing safe drinking water remains a priority in maintaining public health. A number of waterborne outbreaks have been summarised in a book, "Safe Drinking Water-Lessons from Recent Outbreaks in Affluent Nations", by Hrudey and will not be discussed further in this thesis

[6]. As this book illustrates, it is crucial that vigilance in water treatment be maintained because the consequences of neglect can be severe [1]. In 2000, insufficient chlorination of drinking water resulted in seven deaths and over 2300 infections in Walkerton, Ontario [6]. Closer to home, failure to remove *Crytosporidium parvum* oocyts from the drinking water resulted in as many as 7,100 infections in North Battleford, Saskatchewan [6].

Unfortunately, microbially contaminated drinking water is still a significant source of disease in developing countries. In the early 1990s, the Peruvian government decided to reduce chlorine levels in their drinking water as a result of the U.S. Environmental Protection Agency's labeling of disinfection byproducts from the chlorination process as carcinogens [7]. With insufficiently chlorinated drinking water, the seventh pandemic of cholera killed 4700 people within a year in South America [8]. In 1998, the World Health Organization (WHO) reported that over 1 billion people, 800 million of whom live in rural areas did not have an "adequate and safe drinking water supply" [1]. Waterborne diseases, including cholera and typhoid fever, still remain a major cause of death, particularly of children in developing countries.

Drinking water comes from two sources: surface waters (such as rivers and reservoirs) and groundwater. These sources become contaminated through contact with human and animal feces containing pathogenic organisms. Generally, groundwater is at a lower risk for microbial contamination than surface waters [1]. However, groundwater contamination can occur when runoff water from precipitation into streams and rivers enters the soil. Surface waters also become contaminated by runoff from agricultural lands and human activity. To eliminate waterborne disease, it is necessary to break the

"fecal-oral cycle by preventing fecal matter from entering water sources and by treating drinking water to kill the pathogens" [1].

1.2 *Escherichia coli* O157:H7

In 1982, *E. coli* O157:H7 was first recognised as a human pathogen when it was associated with two foodborne outbreaks of bloody diarrhea and abdominal cramps following the consumption of undercooked hamburger [9]. Most *E. coli* bacteria are common harmless inhabitants in the gastrointestinal tract of warm-blooded animals. *E. coli* O157:H7 is pathogenic because of its ability to produce hemorrhagic colitis (HC). HC is characterised by abdominal cramps, fever, diarrhea and dehydration. Although infection is usually self-limiting, the bacterium can cause life-threatening complications, including hemolytic uremic syndrome (HUS) in children, the immunocompromised and the elderly [10]. Progression to HUS occurs in about 10% of HC cases with a fatality rate of 2-10% due to renal failure [11].

E. coli O157:H7 is extremely virulent, compared to other pathogenic bacteria, because it has a very low infective dose, estimated to have a median of 100 cells . The incubation period depends on the host immunity, the size of the inoculum and the stomach pH [12]. Disease can occur within 3 hours and on average occurs 3-4 days following exposure [12]. There are no treatments available for HC, only supportive rehydration therapy.

The primary reservoir of *E. coli* O157:H7 is healthy cattle. Human exposure occurs primarily through the consumption of hamburger due to *E. coli* contamination that occurs in its preparation [12]. *E. coli* O157:H7 can also be found in acidic foods such as apple juice and cider, as well as in dairy products. Food crops can become contaminated

through the use of untreated water for irrigation [13]. *E. coli* O157:H7 is primarily a foodborne pathogen. It is not normally a concern in treated drinking water supplies because it is inactivated by adequate chlorination. However, waterborne outbreaks involving the consumption of inadequately treated drinking water contaminated with human sewage or cattle feces have been documented.

1.3 *Salmonella* Typhi

S. Typhi is the bacterium responsible for typhoid fever. Each year, 16 million cases are reported in developing countries with over 600,000 deaths [14]. Typical clinical symptoms include fever, headache, malaise, nausea, vomiting and diarrhea [15]. Severe typhoid fever is fatal in 50-70% of cases without rehydration and antibiotic therapy [15].

The infective dose of *S.* Typhi is approximately 100,000 organisms through oral ingestion [16]. The incubation period ranges from 1 to 3 weeks, with most clinical manifestations appearing after 7 days [16]. Relapse occurs in 5-10% of patients who have resolved the disease [15]. Interestingly, 1-4% of patients who appear healthy after infection become long-term carriers, excreting *S.* Typhi in their feces throughout their entire life. The most infamous carrier was Mary Mallon, nicknamed "Typhoid Mary," who worked as a cook in upstate New York. She is believed to be responsible for the infection of 47 people, including three fatalities in the early 1900s [17].

Unlike *E. coli* O157:H7, humans are the only known reservoirs of *S.* Typhi. Therefore, infection occurs following contamination of food or water supplies with human fecal matter. *S.* Typhi is killed by chlorination and is not present in adequately treated drinking water.

Today, typhoid fever is almost unheard of in developed countries due to water treatment and good sanitation. However, it remains a concern because of international travel to developing countries where it is often an endemic disease.

1.4 *Vibrio cholerae* O1

In the last 135 years, *V. cholerae* O1 has been responsible for seven pandemics [18]. Most infected individuals have no clinical symptoms or only mild diarrhea [2]. Similarly to *E. coli* O157:H7 and *S.* Typhi the severity of the disease depends on host immunity, the size of the inoculum and the stomach pH, as well as on the host's blood group [19]. Interestingly, individuals with type O blood, as classified by the ABO blood system, are more susceptible to infection than other blood groups [20-22]. This has led to the near extinction of this blood group in some endemic areas [20].

V. cholerae is classified into two groups based on its O antigen. Although there are over 200 O antigens, O1 and O139 are the only identified *Vibrio* serotypes that cause pandemic disease. *V. cholerae* O1 was responsible for the seven previous pandemics. In 1992, O139 bengal was first identified following cholera outbreaks in India and Bangladesh [23, 24]. Today, *V. cholerae* O139 is recognised as an important pathogen likely to cause the coming eighth pandemic of cholera [2].

The infective dose of *V. cholerae* O1 is approximately 10^6-10^{11} cells [19]. The incubation period ranges from 18 hours to 5 days, with an average of 2 to 3 days [2]. Severe disease is characterised by devastating diarrhea and vomiting, resulting in fluid loss of 500-1000 mL/hour [2]. Without treatment, death occurs within a few hours and most deaths occur in the first day of illness [2]. Since fluid loss far exceeds the absorptive capacity of the intestinal cells, treatment is necessary to prevent death. In fact, case

fatality without treatment exceeds 50%, whereas with proper treatment it drops to 1% [2, 25]. Rehydration therapy replaces lost fluids with those of similar electrolyte composition. Treatment has reduced the number of cholera-related deaths by 3 million/year compared to 20 years ago [2].

There are two important reservoirs for *V. cholerae* O1: humans and zooplankton. Infection can occur through the ingestion of food or water contaminated with human feces or vomit, or by the consumption of contaminated seafood. In developing countries, major waterborne outbreaks typically follow storm events. Cholera outbreaks also peak during the warm seasons. For example, in Bangladesh, the two seasonal cholera disease peaks are related to the ability of *V. cholerae* to grow more easily in warm temperatures [26-28].

Cholera outbreaks have also been associated with contaminated shellfish. *V. cholerae* O1 can grow as free living cells in nutrient-rich coastal waters. Shellfish concentrate *V. cholerae* O1 because they are filter feeders and, as such, absorb all particles including bacteria in the water. Humans are exposed to *V. cholerae* O1 contaminated shellfish because uncooked shellfish is considered a delicacy. Since 1973, *V. cholerae* O1 has become endemic in the Gulf of Mexico and causes cases of shellfish-associated cholera every summer [29]. In developed countries, contaminated shellfish and international travel are the typical exposure routes to *V. cholerae* O1 whereas contaminated drinking water exposure is more common in developing countries.

1.5 Enteric Bacteria Virulence Factors

Pathogenesis is a multifactorial process involving numerous bacterial genes that encode virulence factors facilitating the invasion and colonisation of susceptible hosts

[2]. Although they may differ among species of bacteria, there are several common virulence factors.

Without a doubt, the most important virulence factor involved in bacterium-host interaction is the outer membrane of the bacterial cell, the lipopolysaccharide (LPS). LPS has been shown to play a protective role by resisting host-complement-mediated cell death [30, 31]. However, not all bacteria possess LPS. In fact, some only possess a single membrane enveloping the cell. These differences in membranes allow the classification of bacteria into two distinct groups by a Gram stain. Gram-negative cells are stained pink due to the presence of LPS whereas Gram-positive cells with only a single membrane are easily stained blue as a result of both dyes used.

Gram-negative bacteria such as *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1 have three components in their lipopolysaccharide: lipid A, core polysaccharide (PS) and O antigen polysaccharide [31]. The lipid A is anchored in the LPS whereas the core PS and O antigen PS regions project outwards from the cell.

Within the LPS, the O antigen PS is highly specific to each species of bacteria and is therefore used as a means of further classification. Bacteria are divided into serogroups based on their ability to bind with the O-specific antibodies. The O antigen also plays an important role in bacterial cell adhesion, allowing the effective colonisation of host cells [32]. Although adherence to host cells is not well understood, it has been shown that an antibody to LPS blocked the adhesion of *E. coli* O157 to Henle 407 cells, thereby diminishing the virulence of the bacterium [33].

Another important virulence characteristic is the ability to produce attaching and effacing (A/E) lesions on a variety of cell types [34]. Lesions are characterised by the

"degeneration of intestinal epithelial cells, adherence and assembly of cytoskeleton structures within the host cell" [35]. In pathogenic *E. coli*, two proteins are involved in A/E lesions: initimin, an outer membrane protein involved in cell adhesion and the translocated intimin receptor that facilitates its attachment. The genes transcribing these proteins (*eae* and *tir* respectively) are located in a region of the bacterial genome called the locus of enterocyte effacement (LEE) [36]. The proper transcription of these genes is necessary for a bacterium to colonise a host by producing A/E lesions.

Once bacteria have attached to the intestinal cells, they grow and secrete numerous cytotoxins [11]. In many pathogenic bacteria, these cytotoxins are called Shiga toxins. Their mode of action is to attack the 28S ribosomal RNA, thereby inhibiting protein synthesis [37]. In *E. coli*, two Shiga toxins have been identified: Shiga toxin 1 (Stx 1) and Shiga toxin 2 (Stx 2). Although both toxins have a similar structure and mechanism of cell destruction, Stx 2 is more toxic than Stx 1. In fact, Stx 2 is approximately 1000 times more toxic to human renal cells than Stx 1 [38]. Tesh et al. [39] demonstrated that Stx 2 had an approximately 400-fold lower LD_{50} for mice than stx1. It has also been hypothesised that the high yields of Stx 2 in *E. coli* O157 are responsible for its low infective dose and the principal manifestations of HC and HUS [11]. Interestingly, strains producing Stx 1 and Stx 2 cause less serious disease than those producing Stx 2 alone [40, 41].

In *V. cholerae* O1, cholera toxin (CT) is responsible for the clinical manifestation of cholera. CT stimulates adenylate cyclase, which causes the "intestine to secrete watery fluid rich in sodium, bicarbonate and potassium in volumes far exceeding the intestinal absorptive capacity, leading to shock and dehydration" [2].

Flagella (singular: flagellum) are hair-like structures that extend from many bacterial cells [42]. They move in a propeller fashion, allowing a cell to move around in its environment. The presence of a flagellum, whose DNA sequence is contained in the H antigen gene, is advantageous when colonising a host because the bacteria can move to areas of high nutrient content, enabling their growth and reproduction. The term serotype is used to describe a bacterium when both its O and H antigen have been identified. In the case of *E. coli* O157, the H7 antigen is the most commonly isolated HUS-producing strain [11]. In fact, O157 strains producing flagellar antigens other than H7 are considered less pathogenic because they do not produce Shiga toxins or initimin [11]. *V. cholerae* O1 and *S.* Typhi also possess functional flagellar antigens.

At present, it is not known which virulence factors are necessary for a bacterium to cause disease. However, most researchers believe that producing toxins alone is insufficient. In fact, toxin production will only enhance pathogenicity in the presence of other virulence factors [43]. The minimum requirements for pathogenicity of *E. coli* are thought to be the ability to adhere to intestinal cells and to produce Shiga toxin 2 [11].

1.6 Traditional Detection Techniques

1.6.1 Culture

Culture methods are the most commonly used bacterial detection and identification techniques available. They are easy to perform once the growth conditions for specific bacteria have been developed. Bacteria are identified based on their ability to grow on differential media; only certain bacteria present in a sample can metabolise nutrients present in the media. For example, unlike 95% of *E. coli* strains, most *E. coli* O157:H7 strains cannot ferment sorbitol or produce beta-glucuronidase [44]. Therefore,

presumptive identification of *E. coli* O157:H7 is made using sorbitol MacConkey (SMAC) agar that differentiates *E. coli* O157:H7 from other *E. coli* based on its inability to ferment sorbitol. On SMAC, *E. coli* O157:H7 appear as colourless colonies among the pink colonies of sorbitol-fermenting bacteria [45]. Beta-glucuronidase activity can also be evaluated by incorporating 4-methylumbelliferyl-β-D-glucoronide (MUG) in the SMAC medium [46]. The enzyme cleaves MUG to 4-methylumbelliferone (MU) and glucoronide. When exposed to ultraviolet (UV) light (365 nm), fluorescence is emitted by MU on the medium [46, 47].

A positive result on SMAC is only a preliminary identification of *E. coli* O157:H7 because there are other bacteria with the same biochemical characteristics. False positives may result from another organism, *Escherichia hermanni*, that is not only a non-sorbitol fermenter but also a non-beta-glucoronidase producer [48]. In this case, a cellobiose medium can differentiate the bacteria based on the observation that no (0%) *E. coli* O157:H7 will grow on this medium, whereas most (94-97%) *E. hermanni* will [48]. This additional culture step further increases the amount of time required for identification. False negative results also occur because *E. coli* O157:H7 strains have been reported that can ferment sorbitol and produce beta-glucuronidase [45, 47].

V. cholerae O1 ferments glucose, sucrose and mannitol; therefore its identification can be made by analysing a clinical sample on a differential medium. For example, a fecal sample can be streaked onto thiosulphate citrate bile salts sucrose medium (TCBS) that inhibits the growth of normal fecal bacteria and supports the growth of all *Vibrio* species. After a 6-12 hour incubation, a second TCBS plate is streaked with pure colonies from the first plate and incubated for 18-24 hours. On TCBS, *Vibrio*

colonies are smooth and yellow with raised centres [2]. Culture isolation is only the first step in identifying *V. cholerae* O1 colonies among other *Vibrio* strains. In fact, positive identification of *V. cholerae* O1 requires a series of biochemical and immunological tests.

Preliminary identification of *S.* Typhi can be performed by plating on bismuth sulfite agar. The fermentation of glucose by these bacteria reduces sulphite to sulphide that combines with ferric ions to produce ferric sulphide. Following incubation, *S.* Typhi appears as black colonies with a metallic sheen [49].

A further limitation of culture techniques is that they are time-consuming. Most bacteria require incubation periods of 3-7 days before colonies are visible. Also, it is not possible to simultaneously detect and identify multiple pathogens using one differential medium plate because it is difficult to distinguish them based on their colony morphology. Also, since pathogenic bacteria are often present in very low numbers, several plates are often required to streak the entire sample, which further increases the time and cost of analysis [50]. As a result, culture methods have proven to be time-consuming, tedious and lacking specificity [51].

1.6.2 Biochemical

Once pure colonies have been obtained on differential media, biochemical tests are performed to further identify the bacteria. Similarly to differential media, biochemical tests assess the ability of a bacterium to metabolise nutrients into identifiable metabolites. Even though results are obtained quickly, these tests also lack specificity. There are numerous biochemical tests available that evaluate the metabolism of a bacterium. One example, the oxidase test, evaluates the ability of bacteria to catalyse the transport of electrons between donors and a redox dye. Once the redox dye is reduced, it turns deep

purple. Although this test distinguishes *Vibrio* species from most enteric bacteria, it suffers from *E. coli* interference because this bacterium also possesses oxidase enzymes. Lysine and ornithine decarboxylase tests can be used to further distinguish these two bacteria.

1.6.3 Immunological

Immunological methods of detection rely on the specific binding of an antibody to an antigen. Similarly to culture and biochemical tests, they lack specificity because they cannot detect strain specific differences in antigens and cannot be used alone for bacteria identification [45].

Monoclonal antibodies are produced for O antigens, flagellar antigens and cytotoxins. Immunological assays that target bacterial antigens have numerous disadvantages. In fact, cross-reactivity with other bacterial antigens is a common problem that yields false positive results. In the case of *E. coli* O157:H7, O157 LPS antibody cross-reactivity results from the presence of 4-amino-1,6-dideoxy D-mannopyranosyl as a constituent sugar of the LPS in other bacteria [52]. As shown in Figure 1.1, the O157 antibody crossreacts with the LPS of *V. cholerae* O1 and *S.* Typhi because of the presence of the constituent sugar in the antibody binding site [52].

Figure 1.1 *E. coli* O157 antibody cross-reacting with *V. cholerae* O1 and *S.* Typhi antigens

Antibodies for the H7 flagellum and the Shiga toxins also lack specificity because not all *E. coli* O157 family members possess these phenotypic characteristics. In fact, an *E. coli* O157 bacterium may not have a functional flagellum (designated O157:H$^-$) [53]. They may also possess various combinations of Shiga toxins, including Stx 1 alone, Stx 2 alone, both Stx 1 and Stx 2 together or none [45].

Monoclonal antibodies have also been developed for the O1 and O139 antigens of *V. cholerae*. However, false positives result because *Vibrio* colonies grown on TCBS medium can autoagglutinate [2]. Therefore, prior to immunological testing, *Vibrio* colonies should be grown on non-selective media.

The Widal immunological assay is the most widely used assay to identify *S.* Typhi [54]. Antibodies are produced that are specific for the O9 and the Hd antigens of *S.* Typhi. Unfortunately, these antibodies cross-react with antigens from other *Salmonella* species and enteric bacteria [15]. Antibodies for the polysaccharide capsular antigen Vi can distinguish among most species of *Salmonella* but will cross-react with *Salmonella* Hirschfieldii, *Salmonella* Dublin and *Citrobacter freundii* [15].

Another important limitation of immunological assays is that they cannot distinguish between antigens from live (viable) and dead (non-viable) bacteria. Following cell death, a bacterium may not have degraded and therefore may still possess antigens leading to false positive results [55].

In conclusion, traditional detection techniques lack specificity because they rely on phenotypic and biochemical characteristics of bacteria instead of genetic sequences for identification [56]. As a result, these methods are most applicable to preliminary screening prior to extensive characterisation using molecular techniques.

1.7 Molecular Techniques

1.7.1 Polymerase Chain Reaction (PCR)

Molecular techniques such as PCR can amplify unique gene sequences of bacteria. PCR is extremely sensitive because, in theory, it can amplify as little as one molecule of the starting template DNA in the presence of its gene-specific primers. Therefore, PCR is useful in the detection of minute amounts of DNA. It is also highly specific because it amplifies only target DNA in the presence of their complementary primers, allowing cross-reacting bacteria to be distinguished.

The PCR mixture consists of target DNA (also called template DNA), DNA polymerase, all four deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), amplification buffer, $MgCl_2$ and forward and reverse primers that flank the template DNA. PCR is performed in a thermal cycler following a programmed amplification protocol.

Prior to amplification, double-stranded template DNA is denatured to single strands by a 3 minute incubation at 95°C. Next, gene-specific primers anneal their

complementary template sequence after the temperature is lowered to their annealing temperature. DNA polymerase then extends the annealed primers by inserting dNTPs complementary to the template. These three steps: denaturation, annealing and extension, compose one cycle of DNA amplification. In each successive cycle, the amount of template DNA doubles. In fact, in twenty cycles of PCR amplification the amount of DNA increases around one million times (2^n where n is the number of cycles therefore $2^{20} \sim 1{,}000{,}000$) [57].

Although tremendously useful, conventional PCR is limited to the amplification of one template per reaction tube. Therefore, it can become time-consuming and costly when multiple different templates need to be amplified.

1.7.2 Multiplex PCR (MPCR)

Multiplex PCR can be used to simultaneously amplify multiple different templates. MPCR differs from conventional PCR because the amplification reagents, including $MgSO_4$ and buffer, have been optimised to allow several templates to be amplified in one reaction tube and each amplification cycle is slightly longer. MPCR begins with an initial DNA denaturation of 15 minutes, compared to 3 minutes in conventional PCR. The annealing and extension steps are 60 seconds longer than conventional PCR to compensate for the abundance of template and allow the primers extra time to hybridise to their template sequence.

MPCR is advantageous because multiple targets can be amplified in the presence of their gene-specific primers, thereby allowing several pathogenic bacteria to be simultaneously amplified. This feature is useful in analysis when a variety of bacteria are present in a sample or when the bacteria in the sample are unknown.

1.7.3 Limitations of PCR in the Detection of Viable Bacteria

Although PCR has been proven to be a sensitive and specific analytical tool, it cannot discriminate between viable and non-viable bacteria. Detecting viable bacteria is important because they are actively producing virulence factors, including toxins that cause human illness. PCR does not provide viability information because it amplifies all DNA, regardless whether the cells are viable or dead. DNA is not a marker of cell viability because it does not degrade immediately following cell death and can remain intact for several days [58]. In fact, no relationship between viability and PCR detection could be established for *Listeria monocytogenes* and *E. coli* cells that had been killed by high temperature, acidification, hydrogen peroxide, drying or starvation [58]. In another study, cells that had been autoclaved produced DNA products following PCR [59].

To improve discrimination power, a pre-enrichment step is typically performed that allows viable cells to grow and multiply [60]. This added step increases the concentration of DNA produced by viable cells and thereby decreases the potential for false positive results. However, the pre-enrichment process takes between 6-48 hours to complete, becoming the bottleneck for subsequent PCR [61].

1.7.4 mRNA as a Marker of Viability

According to the central dogma of molecular biology, "DNA directs its own replication and transcription to RNA which in turn is translated into proteins" (DNA⇒ RNA⇒ Protein) [57]. There are three types of RNA present in a cell: messenger RNA (mRNA), ribosomal RNA (rRNA) and transfer RNA (tRNA). mRNA is the complementary single-stranded copy of transcribed DNA, whereas rRNA and tRNA are involved in the translation of the information contained in mRNA to a functional protein.

In an *E. coli* cell, mRNA makes up approximately 4% of the total RNA whereas tRNA and rRNA make up 15% and 80%, respectively [62].

Unlike DNA, mRNA has a short half-life in replicating cells, typically on the order of a few minutes [63]. RNA is more susceptible to degradation because of the presence of the 2'-hydroxyl group of ribose [59]. Thus, RNA more accurately reflects the viability of bacteria.

The definition of cell viability is controversial because of the ability of cells to enter dormancy during periods of environmental or nutrient stress [59]. These dormant cells remain viable, but not culturable, and when they return to optimum conditions they will grow and multiply. Therefore, an improved definition suggested by Sheridan et al. [59] is that a viable cell has the ability to replicate under optimum conditions.

A wide variety of viable food pathogens have been detected using mRNA as target: *Salmonella* spp. [64, 65], *Campylobacter* spp. [66], *V. cholerae* O1 [67], *L. monocytogenes* [68] and *E. coli* strains [59, 69]. However, this is still a developing research area because of difficulties in identifying appropriate target mRNA that accurately reflects cell viability.

The most obvious requirement of an mRNA target is that it has a short half-life in dead cells. Not all mRNA degrades at the same rate because the degradation rate depends on the treatment used to kill the cells, as well as on the holding conditions following cell death. Sheridan et al. [59] evaluated the relationship between three *E. coli* mRNA: *rpoH*, *groEL* and *tufA*, and cell viability following heat and ethanol treatments. They found that the mRNA from heat-treated cells (100°C for 5 min, 80°C for 10 min or 60°C for 20 min) became undetectable after 2-16 hours, depending on the mRNA target. In contrast, the

mRNA from ethanol-treated cells (50% ethanol at 23°C for 7 min) was still detectable after 16 h. In another study, Sheridan et al. [69] confirmed their earlier findings by demonstrating that *tufA* mRNA was not detectable in cells killed by autoclaving (121°C for 15 min), boiling (100°C for 10 min) or acidification with HCl (pH 2.0 for 5 min), but it was following ethanol treatment (50% ethanol at 23°C for 7 min). PCR gave positive results for all these samples, but was undetectable following acidification. They further examined the persistence of *tufA* mRNA following ethanol treatment by incubating the sample in liquid medium broth. They concluded that *tufA* degradation was dependent on the holding temperature because mRNA was detectable for up to 16 hours at 15°C and 4°C, but was only detectable for 2 hours at 37°C. The observed variation in degradation according to treatment was attributed to the destruction of RNAses (enzymes capable of degrading RNA). They hypothesised that mRNA degraded more quickly with treatments that did not destroy RNAses. Yaron and Matthews [55] evaluated the *rfbE*, *fliC*, *stx1*, *mobA*, *eae*, *hly* and 16S rRNA genes of *E. coli* O157:H7 to identify an appropriate mRNA target. Cells were killed either by a 60°C water bath for 20 min or by autoclaving (121°C for 15 min). PCR was shown to amplify 16S rRNA and *rfbE*, *fliC* and *stx1* DNA following both treatments. They also found that some mRNA targets were detectable 2 hours following autoclaving but not following the water bath treatment. As a result, they concluded that in addition to inactivating treatment and holding conditions, the target mRNA was also an important factor determining its degradation [69]. Interestingly, mRNA degradation following typical drinking water disinfection treatments that include chlorination or ozonation has not been investigated.

Ideal target mRNA should also be specific to the target pathogen and transcribed in all growth phases and environmental conditions [55]. However, the existence of viable non-culturable cells (VBNC) has complicated the search. Fortunately, some studies have suggested that mRNA produced during the late stationary growth phase may be protected from RNAse degradation depending on the presence of secondary structures, post-transcriptional modifications and associations with ribosomes [69]. Yaron and Matthews identified 16s rRNA, *mobA*, *rfbE* and *stx1* as good *E. coli* O157:H7 viability markers in VBNC cells. They concluded that the *rfbE* gene was the most appropriate viability marker because it was also detectable during all growth phases.

rRNA and tRNA have also been examined as viability markers. Although rRNA is found in all bacterial ribosomes and is present in a high number of copies, it can persist for as long as DNA in the inactivated cells and is not specific to the bacteria species [70, 71]. Not many studies have examined tRNA as a viability marker but Davis et al. [72] found that it persisted even longer than rRNA in *E. coli* cells. As a result, rRNA and tRNA do not appear to be viability markers.

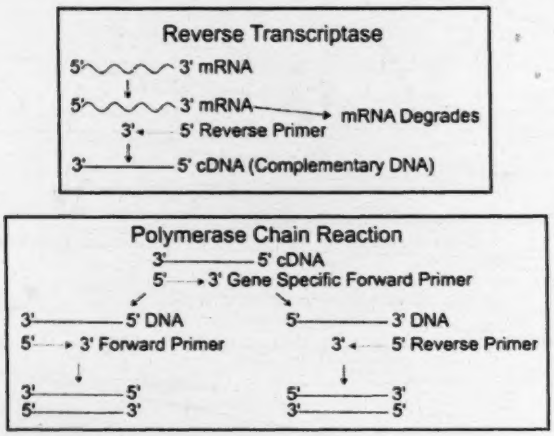
In conclusion, an ideal target mRNA viability marker should be constitutively expressed, species-specific, quickly degraded under a variety of treatments and present in VBNC cells. Given these enormous expectations, it is not surprising that there are difficulties in identifying mRNA viability markers. At present, the choice primarily depends on the targeted bacterial gene and the availability of published studies.

1.7.5 Reverse Transcription-PCR (RT-PCR)

As a consequence of its ability to degrade quicker than DNA, mRNA needs to be converted to complementary DNA (cDNA) prior to analysis. The process reverse

transcription (RT) converts mRNA using a reverse primer to cDNA prior to its degradation. The resulting cDNA is an exact copy of the mRNA sequence with the exception that U nucleotides in the mRNA have been replaced by T nucleotides in the cDNA. The single-stranded cDNA is amplified to double-stranded DNA using gene-specific forward and reverse primers. Both reverse transcription and PCR processes are shown in Figure 1.2. The coupling of RT to PCR can overcome the challenge of detecting viable bacteria [50]. It also decreases the analysis time because it eliminates time-consuming pre-enrichment culture steps.

Figure 1.2 Coupling reverse transcription with polymerase chain reaction



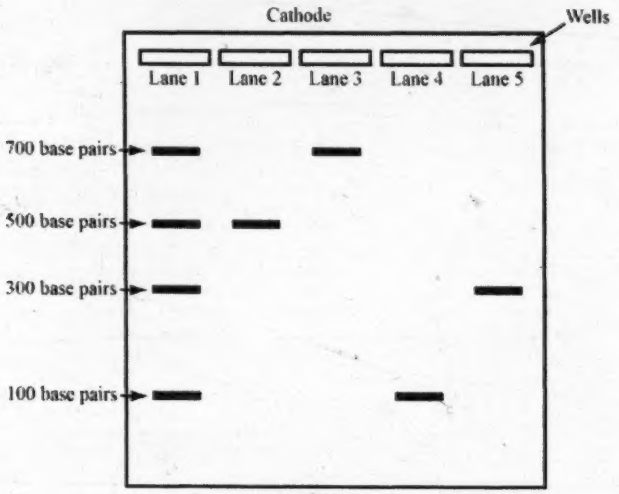
1.7.6 Detection of the PCR Amplified Product

1.7.6.1 Gel Electrophoresis

Gel electrophoresis is widely used to detect PCR products because it is inexpensive and relatively easy to perform. DNA samples mixed with buffer solution are loaded into wells at the cathode end of a gel made of agarose or polyacrylamide. When potential is applied, the negatively charged DNA is repelled by the cathode and migrates through pores in the gel towards the anode at the opposite end. Migration is size-

dependent; larger DNA molecules move slower and take longer to reach the anode, compared to smaller molecules [57]. The separated DNA molecules are detected using ethidium bromide. The DNA molecules intercalate ethidium bromide and are visualised as fluorescent bands under UV light. The sizes of the DNA molecules are identified by comparison with a molecular weight ladder. The process of gel electrophoresis is shown in Figure 1.3.

Figure 1.3 Separation of different sized DNA molecules using gel electrophoresis



Lane 1 - DNA size ladder
Lane 2 - 500 base pair DNA
Lane 3 - 700 base pair DNA
Lane 4 - 100 base pair DNA
Lane 5 - 300 base pair DNA

Agarose gel electrophoresis detection does not provide DNA sequence information because identification is based on the position of the molecules on the gel. Also, non-specific amplification products resulting from primers annealing to mismatch DNA sequences can lead to the presence of unexpected and uncharacterised bands, further complicating analysis; therefore, gel electrophoresis is not specific [73].

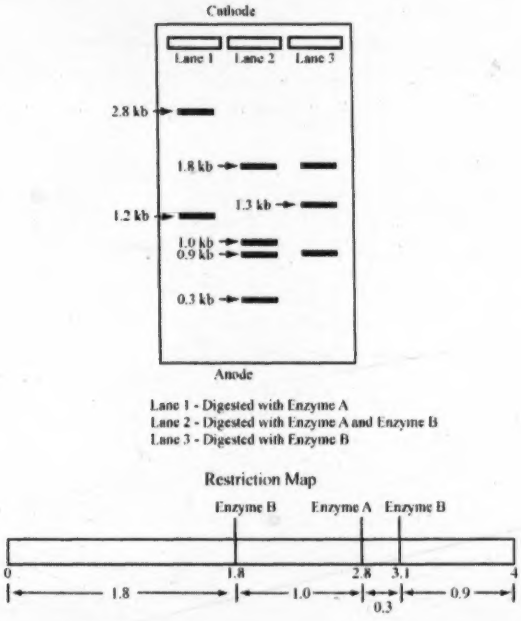
Agarose gel electrophoresis also has limited resolution. It is often not able to separate DNA molecules that differ by fewer than 50 bases. Therefore, PCR primers must be designed to amplify DNA sequences yielding products with a sufficient size difference.

1.7.6.2 Restriction Fragment Linked Polymorphism

Restriction Fragment Linked Polymorphism (RFLP) is useful for analysing large DNA molecules that require processing before they are applied to an agarose gel. Large DNA molecules are first digested by restriction endonucleases to yield smaller fragments. Restriction endonucleases digest DNA at restriction sites within the gene sequence. Restriction sites are unique sequences that act as binding sites for the restriction endonuclease. The number and size of the DNA fragments produced following digestion depends on the number of restriction sites present within a sequence.

Following digestion, the fragments are separated according to their size on an agarose gel. As shown in Figure 1.4, comparing the lengths of the fragments obtained following digestion with different enzymes yields a restriction map. A restriction map details the number and location of unique sequences within a DNA molecule.

Figure 1.4 Separation of digested DNA molecules using gel electrophoresis and the construction of a restriction map



Restriction maps are useful for estimating the degree of difference between related sequences and for gaining minimal sequence information. However, because restriction maps provide only limited sequence information, they cannot identify the location of single nucleotide polymorphisms (SNPs) because they do not change the size of the PCR product.

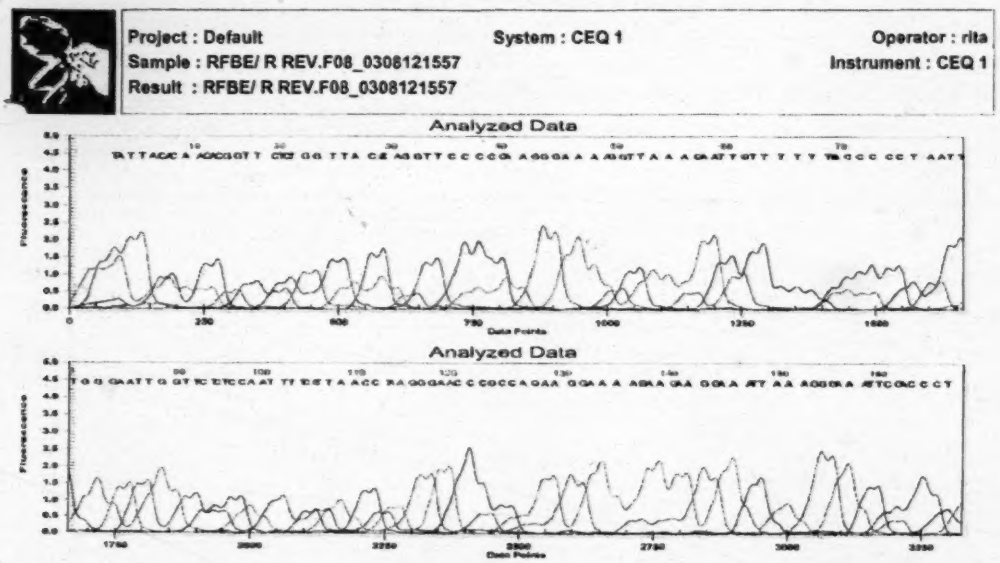
1.7.6.3 DNA Sequencing

The gold standard in the analysis of PCR products is sequencing. DNA sequencing allows the entire nucleotide sequence of the PCR product to be determined.

The most commonly used technique for sequencing is the chain-terminator method. This technique utilises DNA polymerase I (from *E. coli*) to make a complementary strand of the DNA being sequenced [57]. To initiate DNA synthesis, the DNA template, gene-specific primers, all four nucleotides (dNTPs) (one type being α-^{32}P

labelled), DNA polymerase and a small amount of 2',3'-dideoxynucleotide triphosphate (ddNTP) of one base are required [57]. As soon as ddNTP is incorporated into the growing strand instead of the normal dNTP, DNA synthesis is terminated because it has no 3'OH to attach to the strand. Following several reactions, a series of short DNA strands are produced, each terminated by ddNTP at one of the positions occupied by its corresponding dNTP [57]. Sequencing is performed by reacting each of the four ddNTPs in separate tubes. The resulting strands are run on a polyacrylamide gel in parallel lines. The sequencing process is automated and the results are displayed in a graph showing different colored peaks that each correspond to a different nucleotide. The nucleotide is identified by the highest peak at any position in the DNA sequence. The resulting DNA sequence is shown at the top of the graph, as shown in Figure 1.5.

Figure 1.5 Sample sequencing results from the *rfbE* gene of *E. coli* O157:H7



DNA sequencing can be useful when the nucleotide sequence must be known to identify SNPs. However, it is time-consuming because the PCR product must be cloned

into a vector prior to sequencing. Cloning is recommended because it improves efficiency and accuracy of the DNA sequencing. Unfortunately, it substantially increases the analysis time because it takes up to 4 working days (8 hours/day). As a result, DNA sequencing is not practical for routine sample analysis.

1.8 New Detection Techniques

1.8.1 Real-time PCR

It is often desirable to quantify the amount of template DNA present in a sample. With conventional PCR, the amount of DNA is quantified following amplification. Usually, DNA is quantified by visual comparison with a fluorescence intensity marker (i.e. lambda Hind III) after agarose gel electrophoresis, yielding insensitive results.

A newly developed technique called real-time PCR has the ability to quantify template DNA during the amplification process, producing more accurate quantification results [74-77]. Real-time PCR is performed in the same manner as conventional PCR, except that each newly amplified double-stranded DNA molecule is detected through the hybridisation of a complementary fluorescently labelled probe. Analysis time is minimised because the amplification and detection of a template is performed simultaneously [78].

Background fluorescence is controlled by holding unbound probes in a configuration that quenches their fluorescence. Once the probe binds its complementary template sequence, the configuration of the molecule changes and fluorescence is emitted. Fluorescence data is recorded during each successive amplification cycle by the real-time PCR instrument until it reaches the threshold cycle, at which time a detectable fluorescence change occurs [79]. The threshold cycle is proportional to the initial amount

of DNA [76, 80]. Using samples containing 10 times dilutions, a calibration curve can be constructed allowing unknown samples to be quantified [74, 76, 78].

There are four types of fluorescently labelled probes that can be used in real-time PCR: double-stranded DNA binding dyes, TaqMan probes, molecular beacons (MBs) and scorpions [79]. Double-stranded DNA binding dyes are the least expensive probes currently available [74]. They quantify template DNA by preferentially binding only double-stranded DNA present in solution. However, these dyes lack specificity because, in addition to newly synthesised DNA, they also bind to non-specific amplifications and primer-dimer complexes [74, 76, 77]. This leads to an overestimation of the initial amount of template DNA [74]. Also, these dyes can only be used in single-plex reactions (reactions with only one type of template DNA) because there is no way to distinguish between different templates.

TaqMan probes consist of a fluorescent dye at the 5' end and a quencher at the 3' end. The TaqMan probe binds its complementary sequence on the initial template DNA prior to amplification. No background fluorescence is emitted because, in this configuration, the fluorescent molecule and quencher are in close proximity. Fluorescence is emitted once the DNA polymerase replicates the template DNA because its exonuclease activity cleaves the TaqMan probe at the 3' end, releasing the quencher molecule. With no quencher present, fluorescence is emitted and detected by the real-time PCR instrument. Although more specific, TaqMan quantification is indirect and therefore, not as accurate as direct detection [78].

Molecular beacons offer more direct quantification of template DNA, compared to TaqMan [81]. Similarly to TaqMan, MBs have a fluorescent molecule at one end and

its quencher at the opposite end. The two probes differ with respect to configuration, with the MB adopting a hairpin rather than a linear configuration. The hairpin is complementary to a nucleotide sequence in a template DNA, while the stem is formed by the annealing of the sequences adjacent to the hairpin [75]. In the hairpin configuration, the fluorescent dye is directly adjacent to the quencher so that no fluorescence is emitted. The configuration changes from hairpin to linear when the MB binds to template DNA and emits fluorescence. MBs are very specific because the linear configuration only occurs in the presence of its exact complementary template sequence as a consequence of the hairpin configuration being more energetically favourable [74]. Given this characteristic, MBs are useful for detecting SNPs in single-plex reactions [74, 80]. MBs can also be used in multiplex reactions when each MB is labelled with a fluorescent molecule that emits at different wavelengths, allowing the distinction of multiple templates [80]. Extensive multiplexing with MBs can be challenging because the data must be adjusted to take into "account differing dye intensities and spectral cross-talk problems" [81]. Also, there are presently only six different fluorescent molecules commercially available due to the limited availability of "non-spectral overlapping fluorescent molecules and the spectra of the detection lasers used" [61, 62]. Therefore, their usefulness in the analysis of clinical and water samples is limited because there is often a variety of bacteria present. Finally, the specificity of the MB probe can be compromised when the annealing temperature is not optimised, resulting in the detection of mismatch templates [79].

Scorpions offer the greatest specificity and sensitivity of all real-time PCR probes. Unlike the other probes, scorpions consist of a PCR primer that is covalently linked to a

MB at its 5' end, allowing the amplification and detection of template in one step [76, 82]. They are more specific compared to MBs because the probe is attached to the PCR primer holding it close to the template DNA. Also, they do not rely on a chance meeting between probes present at a low concentration and the template DNA [76, 82]. As a result, scorpions allow for more rapid PCR cycling times and generate stronger fluorescence signal strengths compared to TaqMan and MBs [82].

Unfortunately, scorpions are the most expensive probes available and therefore have limited use in clinical settings. Similarly to MBs, they are also limited by their ability to simultaneously detect only six different DNA templates [75, 83].

Although real-time PCR with fluorescent probes is promising, its specificity can be seriously compromised if sub-optimal assay conditions are used. Factors affecting the ability of the assay to detect SNPs include the length of the probe, the position of the mismatch within the probe, the temperature at which fluorescence is read and the $MgCl_2$ concentration [75, 79].

1.8.2 DNA Microarray Technology

1.8.2.1 Introduction

In 1995, Schena et al. [84] first reported the use of microarray technology in gene expression studies. Since then, the transcription levels of numerous genes have been analysed under environmental conditions [85-87], in disease states [88, 89] and in the presence of various toxic compounds [90], therapeutic compounds and antibiotics [45, 91, 92]. However, it was not until recently that researchers began to investigate its potential for genotypic characterisation and identification of various pathogenic bacteria.

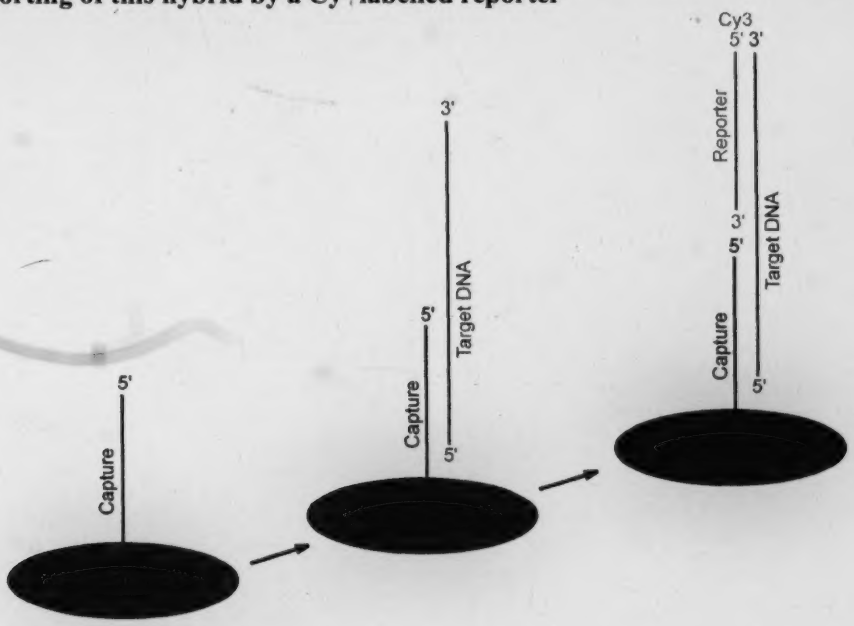
Similar to PCR, DNA microarray technology utilises the preferential hydrogen bonding between complementary nucleic acids in DNA [93]. There are four nucleic acids (commonly called bases) in DNA: adenine (A), guanine (G), thymine (T) and cytosine (C). Adenine preferentially hybridises with its complementary base, thymine, through two hydrogen bonds, whereas guanine preferentially hybridises with its complementary base, cytosine, through three hydrogen bonds. It is the strength of hydrogen bonding that holds the two linear strands of DNA together in a double helix.

In DNA, nucleotides are ordered into specific sequences in genes that dictate a specific cellular function. Interestingly, genes dictating the same cellular function may differ in nucleotide sequence between two strains of bacteria. As a result, the nucleotide sequence contained within a gene can be absolutely specific for a target bacterium. Gene sequences can be obtained through GenBank, a publicly available database containing published gene sequences. Within Genbank (www.ncbi.nlm.nih.gov), genes are organised by accession numbers. There can be numerous accession numbers for a particular gene sequence inputted by various researchers; therefore, consistency in sequence must first be analysed using an alignment program.

Although there are variations, most DNA arrays are designed with a similar format. First, an oligonucleotide (also called a capture) is designed to be complementary to the targeted gene sequence. To facilitate hybridisation, the complementary oligonucleotide is immobilised on a solid surface at a specific location through a biotin molecule attached to its 3' end. The single-stranded target gene sequences are applied to the capture spots and allowed to hybridise. For hybridisation to occur, a substantial portion of the targeted sequence must match the capture sequence. A fluorescently

labelled oligonucleotide (called reporter) that is complementary to the targeted gene sequence is added to the array. Identification is based on the fluorescence intensity and its location on the microarray. The microarray hybridisation sequence is shown in Figure 1.6.

Figure 1.6 The addressing of capture and target to a test site on the microarray and the reporting of this hybrid by a Cy^3 labelled reporter



Unlike real-time PCR, the template DNA must be produced in a separate PCR reaction prior to its detection. It must also be denatured to single strands to facilitate hybridisation to its complementary capture.

Array technology is advantageous because it uses these "nucleic acid signatures" and therefore has the potential of increasing specificity beyond traditional detection techniques [47]. Specificity is very important when analysing bacterial samples because there are many sample matrices where a small number of pathogenic bacteria coexist

with large numbers of non-pathogenic bacteria [94]. Such matrices include food, water, soil and clinical samples.

Another advantage is the ability to obtain simultaneous sequence information from many different genes in one experiment. The high-throughput processing of arrays is attained because they permit simultaneous hybridisation of hundreds to thousands of target gene sequences on a single substrate [95, 96]. Therefore, it is ideal for an extensive genetic profile of a bacterial strain that includes "phylogenetic classification, specific biochemical" and virulence markers [73]. It also allows rapid multiplexed detection of a strain among many possible alternatives [47]. Numerous traditional techniques would be required to obtain a comparable amount of information from the same sample. In fact, traditional techniques provide limited information about all the genes that are present within a bacterial genome [56]. Because they do not rely on phenotypic expression for identification, arrays can be useful for screening non-expressed genes [45].

1.8.2.2 Types of Microarrays

The two main types of microarrays differ primarily in the process of hybridisation. In passive addressing microarrays oligonucleotides are manually spotted onto test sites on the array. There is no physical process driving the addressing of the capture to the test site and the addressing of the target to capture loaded sites. Electronic addressing microarrays rely on electronic activation of individual test sites to address captures and targets to activated test sites.

As a result of the hybridisation process, passive hybridisation microarrays can accommodate thousands of capture sites per substrate whereas electronic hybridisation microarrays can presently only accommodate 100 capture sites.

In passive arrays, captures are deposited on glass because of its low fluorescence, cost and transparency. Glass substrates possess a modified surface chemistry that allows the attachment of modified captures to a chemically reactive glass surface. Glass surfaces can be modified to contain amino, epoxide, carboxylic acid and aldehyde functional groups [97]. Captures are immobilised to the glass surface through the attachment of a functional group to their 5' or 3' end. Target gene sequences are then added to the glass substrate and allowed to passively hybridise to their complementary capture. Typically, the targets are fluorescently labelled, thereby simplifying the detection process because a reporter is not required.

Passive arrays are advantageous because they can accommodate thousands of spotted oligonucleotides and, as a result, a multitude of genes can be analysed per sample [98]. Also, the substrate used, glass, is relatively inexpensive to purchase compared to the substrate used in low density arrays.

Unfortunately, they possess many disadvantages limiting their use in a high-throughput environment, as shown in Table 1.1. The major disadvantage is the passive hybridisation between the capture and target sequences. Because there is no physical process driving the hybridisation, a lengthy hybridisation period is often required to get a detectable fluorescence signal [99]. Depending on the fluorescence intensity requirements, hybridisation can take anywhere from 3 hours to overnight and, as a result, it increases the analysis time substantially [62]. A further limitation of passive hybridisation is that analysis of many different samples cannot be simultaneously performed [99]. Hybridisation results would be difficult to interpret because targets are not being applied to known captures sites.

Table 1.1 Advantages and disadvantages of passive and electronic hybridisation microarrays

Passive Hybridisation Arrays		Electronic Hybridisation Arrays	
Advantage	**Disadvantage**	**Advantage**	**Disadvantage**
Can accommodate thousands of oligonucleotides	Lengthy passive hybridisation	Short electronic hybridisation	Can only accommodate 100 oligonucleotides
Inexpensive substrate	Not amenable to simultaneous analysis of many different samples	Amenable to simultaneous analysis of many different samples	More expensive substrate than glass
	High background fluorescence	Test sites individually connected to electrodes	
		Automated; therefore decreasing human error and increasing reliability between runs	

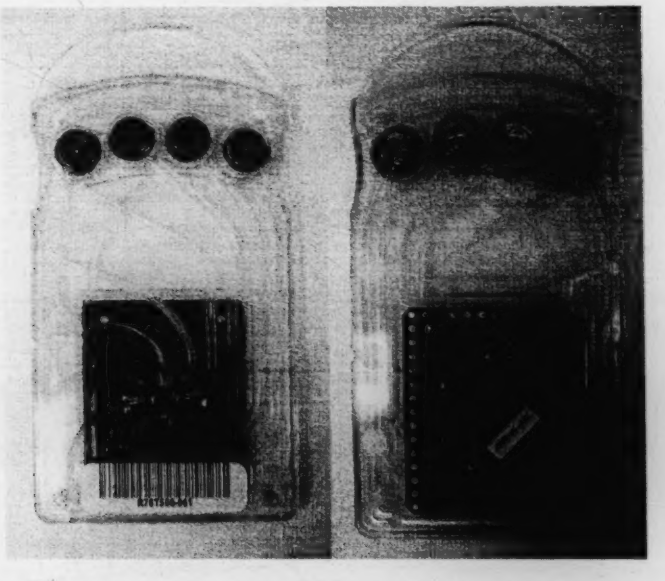
Passive arrays typically have a high background fluorescence reading. This occurs when a fluorescently labelled target binds to the glass instead of its complementary capture. Background fluorescence can be decreased using modified surface chemistries. Another option, Denhardt's solution, is a commercially available mixture of blocking agents that is capable of saturating non-specific binding sites. These methods decrease the background fluorescence, but it remains a serious concern with glass substrates.

Finally, hybridisation can occur between a capture and a mismatch target. This cross-reactivity can occur when there are only a few complementary nucleotides shared between the capture and the target. To remove mismatch hybrids, stringency conditions, such as low salt washes, need to be applied. However, with glass substrates, washing is done manually and therefore it is inefficient and lacks consistency between substrates. Remaining mismatches increase the potential for false positive results.

For passive arrays to have applicability in a high-throughput setting, they would require shorter incubation times, decreased background fluorescence signals and lower cross-reactivities [56].

Current electronic hybridisation arrays consist of 100 individual test sites for capture binding. A low density array produced by Nanogen (San Diego, CA, USA), shown in Figure 1.7, was used in the thesis work presented here. The entire array surface is covered in a hydrogel permeation layer containing streptavidin. To facilitate binding, captures are labelled with biotin at their 3' end. The streptavidin-biotin bond irreversibly attaches the capture to the test site. Each test site is individually connected to an electrode. Voltage is applied to the test site to move and concentrate negatively charged molecules such as captures and target DNA. Electronic hybridisation facilitates the hybridisation of a capture to the array surface and a target to its complementary capture. As compared to passive hybridisation, electronic hybridisation increases the rate of hybridisation, allowing completion in minutes instead of hours [56]. Also, since individual test sites can be electronically activated, conditions are favourable for hybridisation to occur only at those array sites. As a result, multiple samples can be analysed on the same array, enabling side by side comparisons [98].

Figure 1.7 The Nanogen DNA microarray cartridge containing 100 individual test sites



Electronic hybridisation is also advantageous for genetic analysis because it offers greater flexibility in assay configuration, while reducing experimental variation arising from comparisons between arrays [100]. Due to the absence of free cations in buffers used for hybridisation (allowing for DNA phosphate backbone shielding), denatured DNA will remain single-stranded until it reaches the test site, resulting in minimal loss of fluorescence intensity [99].

Finally, electronic arrays are reusable. Because the capture is bound irreversibly, it will not be removed when the targets are removed prior to another analysis. In fact, capture-bound test sites can be reused several times with minimal loss in fluorescence intensity.

Electronic arrays are operated within an automated computer-controlled workstation. The electronic DNA microarray workstation used in this thesis research was also manufactured by Nanogen (San Diego, CA, USA). The workstation is easy to use

because all microarray parameters are operated through a computer interface. The advantages of automation are decreased potential for human error and reduced labour costs associated with each analysis.

As with all technologies, microarrays are not without their disadvantages. Firstly, this technology has yet to be proven accurate for a quantitative analysis of target gene sequences. It is primarily qualitative because of variations in hybridisation efficiency and the lack of internal controls [101]. As previously discussed, real-time PCR would be better suited for quantification.

Another disadvantage is the cost of analysis compared to agarose gel electrophoresis. As shown in Table 1.2, automated DNA microarray analysis is ten times more expensive than agarose gel electrophoresis. However, it is important to evaluate the increased cost within the context of its many advantages. In fact, the increased specificity, sensitivity and high-throughput analysis afforded by microarray analysis outweigh its cost. Also, in high-throughput analysis, agarose gel electrophoresis may be more expensive due to the cost of labour in loading and running the seven separate gels necessary to process 100 samples, compared to one run on the array. Running several gels also increases the potential for mistakes, therefore further increasing the cost of agarose gel electrophoresis.

Table 1.2 Comparison of the cost of agarose gel electrophoresis and automated DNA microarray technology

	Agarose Gel Electrophoresis	Automated DNA Microarray Analysis
Reagents	Ethidium Bromide: $0.96	Capture: $0.80 Reporter: $0.80
Platform	Agarose Gel: $1.50[1]	Cartridge: $260.00[2]
Samples/Platform	14	100
Total Cost/Sample	$0.20	$2.61

[1]Labor costs of running 7 separate gels not included.
[2]Capture loaded sites can be reused further decreasing the analysis cost.

Of all the detection techniques currently available, the DNA microarray can simultaneously analyse the most samples in a timely manner. As shown in Table 1.3, when compared with agarose gel electrophoresis, sample analysis time is decreased. DNA microarray analysis also saves significantly in preparation time compared to loading multiple gels.

Table 1.3 Comparison of the speed of analysis of agarose gel electrophoresis and automated DNA microarray technology

	Agarose Gel Electrophoresis	Automated DNA Microarray Analysis
Time/Run	150 V: 90 min[1]	Capture Addressing: 100 x 1 min Target Addressing: 100 x 4 min Reporter Hybridisation: 1 x 3 min Optics Calibration: 1 x 2 min Reporting: 100 x 2 s
Samples/Run	14	100
Time/Sample	6 min 26 s	5 min 8 s

[1]No value assigned to the length of time required to manually load samples

1.9 Thesis Objectives

The objective of this thesis was to develop a sensitive and specific assay for the detection of viable *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1. In order to achieve this goal, a number of important issues had to be addressed. The steps involved in addressing these issues are described below.

1. Viability markers for the target bacteria had to be selected based on published studies. Information on bacterial gene sequences in GenBank is limited and often the sequences have been obtained without validation. Therefore, in order to confirm the gene sequence of each target bacterium, they were sequenced after cloning and compared with other available sequences in GenBank.
2. Procedures for the extraction of RNA from bacteria and for reverse transcription to convert RNA to cDNA needed to be established and tested.
3. Specific PCR primers had to be designed to amplify unique target bacterial gene sequences and then verified to produce the expected size PCR product using a traditional method, agarose gel electrophoresis.
4. The functionality of the primers had to be confirmed using RT-PCR and the new RT-MPCR assay.
5. Following the development of the RT-MPCR assay, the detection limit and the specificity had to be determined as well the reliability of the assay had to be evaluated using blinded RNA samples as well as bacteria from clinical specimens.
6. The developed RT-MPCR assay was combined with DNA microarray detection techniques to improve the detection limit and the sample throughput.

7. The important parameters in the DNA microarray detection technique, including sample addressing parameters, were investigated.
8. The sensitivity and specificity of the DNA microarray assay for detecting *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1 target genes was studied.

1.10 Thesis Scope

This thesis consists of four chapters. In Chapter 1, a literature review of relevant bacteria detection techniques is presented. Chapter 2 presents the development of a RT-MPCR assay. Chapter 3 documents the initial development of a DNA microarray for the detection of *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1, including sample addressing parameters, detection limit and preliminary specificity experiments. The potential for future research to be expanded from this thesis work is discussed in Chapter 4.

Chapter 2: Development of a Reverse Transcription MPCR Assay for the Simultaneous Detection of *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1

2.1 Introduction

Escherichia coli O157:H7, *S.* Typhi and *V. cholerae* O1 can cause serious gastrointestinal illness. These bacteria are commonly found in untreated and ineffectively treated sewage and water, and pose a threat to public health. Because of the ubiquitous and virulent nature of these bacterial pathogens, there has been a tremendous amount of research in developing specific and rapid detection techniques.

Traditional detection techniques involve culturing of the bacteria, biochemical tests or immunological assays [102]. Culture techniques distinguish bacteria by their ability to grow on differential media. However, positive identification cannot be confirmed by growth on a selective medium alone because many enteric bacteria possess similar biochemical characteristics. A second culture medium is often used for confirmation; therefore, culture techniques are time-consuming and lack specificity. Similarly to culture techniques, biochemical tests evaluate the metabolism of nutrients by bacteria; therefore, they also lack specificity. Immunological assays identify bacteria through the specific binding of an antibody to a bacterial antigen. Although antibodies are usually specific, cross-reactions can occur, compromising specificity [103].

Molecular techniques such as polymerase chain reaction (PCR) have proven to be an invaluable tool for the detection of pathogens [60]. In PCR, gene-specific primers are designed to selectively amplify a target gene, allowing cross-reacting bacteria to be distinguished. When multiple target genes need to be amplified, multiplex PCR (MPCR)

can be performed, which provides a simple tool for the simultaneous detection of multiple pathogenic bacteria [104].

PCR does not provide information related to cell viability because it cannot distinguish between the DNA from viable and non-viable cells [58, 105]. In fact, dead cells may contain amplifiable DNA; therefore, a positive result obtained by PCR does not conclusively demonstrate that viable target cells are present in a sample [50].

Messenger RNA (mRNA) can be used as a marker of viability because it is only present in replicating cells and degrades quickly after cell death [59]. As a result, mRNA can provide information reflecting the viability of the cells in a sample more accurately than can DNA [50]. Several studies have investigated mRNA as a viability marker for bacteria [67, 106, 107].

This chapter details the development and validation of a reverse transcription MPCR assay for the simultaneous detection of viable *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1. The coupling of RT and MPCR avoids time-consuming pre-enrichment and directly detects viable bacteria. MPCR methods for detecting several genes of these bacteria have been previously reported for *E. coli* O157:H7 [51, 60, 61, 103, 104, 108-111], *Salmonella* species [60, 104, 108, 112-114] and *V. cholerae* O1 and O139 [60, 108, 115, 116]. However, there has been no MPCR method developed that simultaneously detects all three viable pathogens present in the same sample. The ability to simultaneously detect these pathogens would be very advantageous, resulting in faster analysis and lower cost.

2.2 Materials and Methods

2.2.1 Safety Precautions

Care was taken while working with *V. cholerae* O1, *S.* Typhi and *E. coli* O157:H7 cultures. All three bacteria are level II pathogens and therefore pose a moderate human health risk to healthy adults. Bacteria growth and RNA extraction was performed in a biosafety hood while I was wearing a lab coat, neoprene gloves and goggles. Spills were immediately cleaned with 70% ethanol.

2.2.2 Bacteria and Growth Conditions

E. coli O157:H7, *V. cholerae* O1 and *S.* Typhi were obtained from Dr. Glen Armstrong at the Enterobacteria Culture Collection, Department of Medical Microbiology and Immunology, University of Alberta, Canada. All bacteria were previously identified using differential media plates. Frozen *E. coli* O157:H7, *S.* Typhi, and *V. cholerae* O1 were streaked on tryptic soy agar, 5% sheep blood tryptic soy agar and nutrient agar plates, respectively, and incubated at 37°C overnight. The agar reagents, with the exception of the sheep blood, were purchased in powder form from Fisher Scientific (Nepean, ON, Canada). A negative control consisting of a non-streaked plate was included for each bacterium. Single colonies were inoculated in 5 mL of broth: tryptic soy broth for *E. coli* O157:H7, brain heart yeast extract broth for *S.* Typhi and nutrient broth for *V. cholerae* O1, along with negative controls (broth alone) at 37°C shaking at 125 rpm overnight. The 5 mL cultures were separately transferred into 50 mL of broth and incubated at 37°C with shaking at 125 rpm until log phase. The cells were harvested in late log phase ($OD_{615} = 0.7$). To confirm that only mRNA and not DNA was

amplified, I included a dead cell control from 1 mL of *E. coli* O157:H7 cells (approximately 10^7cells) that were boiled for 10 min. All cell suspensions were pelleted using a micromax RF centrifuge (Thermo IEC, Needham Heights, MA, USA).

2.2.3 Clinical Bacteria Samples

Non-pathogenic *E. coli* (ATCC 25922), *E. coli* O157:H7, *Listeria monocytogenes*, *Yersinia enterocolitica* and *S.* Typhi were obtained from patient stool samples and serotyped by the Provincial Laboratory of Public Health (Microbiology) at the University of Alberta. Each bacterium was grown in 1 mL liquid media broth (LB) (Fisher Scientific, Nepean, ON, Canada) in a 37°C incubator overnight. The following day, each bacterial culture was determined to have approximately 10^7 cells using a BioRad SmartSpec spectrophotometer (Foster City, CA, USA). Two groups of bacteria were prepared in sterile phosphate buffer solution (PBS) (130 mM NaCl, 10 mM sodium phosphate buffer, pH 7.4). Group 1 contained five bacteria: non-pathogenic *E. coli*, *E. coli* O157:H7, *L. monocytogenes*, *Y. enterocolitica* and *S.* Typhi. No clinical sample of *V. cholerae* O1 was available for inclusion in this experiment. Group 2 contained only non-pathogenic *E. coli*, *L. monocytogenes* and *Y. enterocolitica*. This group was included to demonstrate the specificity of the primers for the *rfbE* and *fliC* genes of *E. coli* O157:H7 and the *tyv* gene of *S.* Typhi. For each group, five serial dilutions (1000 times, 10 times, 10 times, 10 times and 10 times) were performed to obtain samples containing various numbers of bacterial cells. Total RNA was extracted separately from these samples and analysed by the RT-MPCR assay.

2.2.4 Total RNA Extraction

Trizol (Invitrogen Life Technologies, Carlsbad, CA, USA) was used to extract total RNA from the cell pellets. The concentration and purity of the extracted RNA was measured using a BioRad SmartSpec spectrophotometer. According to the Trizol recommended protocol, a ratio of A260/A280 greater than 1.6 indicated that the extracted RNA sample contained minimum protein contamination. All RNA extracts were determined to have a ratio greater than 1.6. The RNA samples were immediately frozen at -70°C until use.

2.2.5 Primer Design

Primers were designed for the *E. coli* O157:H7 *rfbE* and *fliC* genes, *V. cholerae* O1 *rfbE* gene and *S.* Typhi *tyv* gene, as shown in Table 2.1. Forward and reverse primer pairs were designed to be specific only for the intended gene sequence. Specificity was important because all three lipopolysaccharide genes are similar in nucleotide sequence, as shown in Figure 2.1. The resulting primer design was analysed using a basic local alignment search tool (BLAST) of available gene sequences on GenBank (www.ncbi.nlm.nih.gov).

Table 2.1 Sequences and T_m of primer pairs for *E. coli* O157:H7, *V. cholerae* O1 and *S.* Typhi

Target Bacteria	Target Gene and Accession Number	Gene Sequence Position 5'⇒ 3' Strand	PCR Product Size (bp)	Primer Sequence 5'⇒ 3'	T_m (°C)
E. coli O157:H7	*rfbE:* AE005429	7441-7680	239	F gcgcgaattcgtgcttttgatatttttccgagtacattgg	59
				R gcgcgaattctttatatcacgaaaacgtgaaattgctgat	58
E. coli O157:H7	*fliC:* AE005415.1	1981-2160	179	F gcgcgaattcgttggtcgttgcagaaccagcactggtagt	68
				R gcgcgaattcgccgactatacagtctcttacagcgt	61
V. cholerae O1	*rfbE:* X59554	5641-6051	419	F gcgcgaattctatattgatcgcttcgaaactgagtttgcg	61
				R gcgcgaattcatcgccaaatgtacctacttttttgccatt	62
S. Typhi	*tyv:* M29682	1201-1510	329	F gcgcgaattcagcttttaattaccggtggatgtggcttcc	65
				R gcgcgaattcgccgtactgcctcaagtaaatttaaagttc	60

Figure 2.1 Sequence alignments of the lipopolysaccharide genes of *E. coli* O157:H7, *V. cholerae* O1 and *S.* Typhi

a) Alignment of the *rfbE* gene sequences of *E. coli* O157:H7 (Line 1) and *V. cholerae* O1 (Line 2) (5'⇒ 3')

Note: a dash represents no nucleotide match and an asterisk represents a perfect nucleotide match

```
Line 1      ------------------------------------------------------------------------
Line 2      TATATTGATCGCTTCGAAACTGAGTTTGCGGAGTTTTTAAAAGTAAAGCACGCCACAACAGTATCTA

Line 1      ---------GTGCTTTTGATATTTTTCCGAGTACATTGGCATCGTGTGGACAGGGTAAAAAACTGGC
Line 2      ATGGAACAGTTGCGCTACATTTGGCAATGAGCGCGTTGGGAATAACT---CAAGGCGATGAAGTGAT
                     ***  *  ** *      ***  * **** *     *   ** **  *  ** **

Line 1      CTTGTTTCGATGAGTTTAT-CTGCA--AGGTGATTCCTTAATTCC-TCTCTTTCCTCTGCGGTCCTA
Line 2      TGTACCAACATTCACTTATGTTGCCTCGGTTAATACCATAGTCCAGTGTGGTGCGTTACCCGTTTTT
              *      **    ****  ***    * * ** ** ** * *  * *  * * *   * **  *

Line 1      GTTAGAAT------TGAGACCATCCAA-TAAGTGTGAAAAACATCTTTACTTTCCTTGTGGACTTGT
Line 2      GCTGAAATCGAAGGTGAGTCTCTACAAGTGAGCGTAGAGGACGT-TAAACGTAAAATAAATAAAAAG
            * *  ***     **** *  * *** * ** **  *  ** * *  ** *    *    *

Line 1      ACAAGACTGTTGATATTTTTTTTATAAATATCAGCAATTTCACGTT-TTCGTGATATAAA-------
Line 2      ACAAAAGCAGTTATGGCCGTTC-ACATATATGGACAAGCTTGCGATATTCAATCTTTAAGAGATCTG
            **** *    * **      **  * * ****   ***  *  ** * ***    * ***

Line 1      ------------------------------------------------------------------------
Line 2      TGTGATGAGCAGGGCCTCTATTTAATTGAAGACTGTGCAGAAGCAATAGGTACTGCTGTTAATGGCA

Line 1      ------------------------
Line 2      AAAAAGTAGGTACATTTGGCGAT
```

b) Alignment of the *rfbE* gene sequence of *E. coli* O157:H7 (Line 1) and the *tyv* gene sequence of *S.* Typhi (Line 2) (5'⇒ 3')

```
Line 1      ----------GTGCTTTTGATATTTTTCCGAGTACATTGGCATCGTGTGGACAGG
Line 2      AGCTTTTAATTACCGGTGGATGTGGCTTCCTTGGGAGTAATCTTGCCTCCTTTGCTTTAA
                      ***  * **   *  **  *****   * ** ** * **

Line 1      GTAAAAAACTGGCCTTGTTTCGATGAGTTTATCTGCAAGGTGATTCCTTAATTCCTCTCT
Line 2      GTCAAGGGATTGATTTAATTGTATTCGATAATCTATCACGTAAAGGTGCAACAGATAATT
            ** **    * *  **  **  **  * * ****   * ** *      **    *  *

Line 1      TTCCTCTGCGGTCCTAGTTAGAA----TTGAGACCATCCA-------ATAAGTGTGAAAA
Line 2      TACATTGGTTATCCTCCTTAGGAAACTTTGAGTTTGTACATGGTGATATTCGCAACAAAA
            * * *  *   ****  **** *    *****    * **        **  *    ****

Line 1      ACATCTTTACTTTCCTTGTGGACTTGTACAAGACTGTTGATATTTTTTTTAT--------
Line 2      ATGATGTTACAAGATTAATAACTAAGTATATGCCTGATAGCTGTTTTCATCTTGCAGGTC
            *     ****     *  *      *** * * *** *     ****  * *

Line 1      AAATATCAGCAATTTCACGTTTTCGTGATATAAA--------------------------
Line 2      AAGTGGCAATGACTACATCTATTGACAATCCTTGTATGGATTTTGAAATTAATGTAGGTG
            ** *  **   * * **  * **    **

Line 1      ------------------------------
Line 2      GAACTTTAAATTTACTTGAGGCAGTACGGC
```

c) Alignment of the *rfbE* gene sequence of *V. cholerae* O1 (Line 1) and the *tyv* gene sequence of *S.* Typhi (Line 2) (5'⇒ 3')

```
Line 1      TATATTGATCGCTTCGAAACTGAGTTTGCGGAGTTTTTAAAAGTAAAGCACGCCACAACA
Line 2      ------------------------------------------------------------A
                                                                         *

Line 1      GTATCTAATGG--AACAGTTGCGCTACATTTGGCAATGAGCGCGTTGGGAATAACTCAAG
Line 2      GCTTTTAATTACCGGTGGATGTGGCTTCCTTGGGAGTAATCTTGCCTCCTTTGCTTTAAG
            *  * ****        * ** *      **** * * * *  *       *   * ***

Line 1      GCGATGAAGTGATTGTACCAACATTCACTTATGTTGCCTCGGTTAATACCATAGTCCAGT
Line 2      TCAAGGGATTGATT-TAATTGTATTCGATAATCTATCACGTAAAGGTGCAACAGATAATT
             * * * * ***** **     ****  * ** *  *         * * * **   * *

Line 1      GTGGTGCGTTACCCGTTTTTGCTGAAATCGAAGGTGAGTCTCTACAAGTGAGCGTAGAGG
Line 2      TACATTGGTTATCCTCCTTAG--GAAACTTTGAGTTTGTACATGGTGATATTCGCAACAA
                * **** **   ** *  ****      **  **   *     *   ** *

Line 1      ACGTTAAAC-GTAAAATAAATAAAAAGACAAAAGCAGTTATGGCCGTTCACATAT-ATGG
Line 2      AAATGATGTTACAAGATTAATAACTAAGTATATGCC-TGATAGCTGTTTTCATCTTGCAG
            *  * *      ** **_***** *   * * **  * ** ** ***  *** *    *

Line 1      ACAAGCTTGCGATATTCA-ATCTTTAAGAGATCTGTGTGATGAGCACGGCCTCTATTTAA
Line 2      GTCAAGTGGCAATGACTACATCTATTGACAATCCTTGTATGGATTTTGAAATTAATGTAG
              * * ** **    * **** *     ***  ***   **    *   * ** **
```

Primers were also analysed for melting temperature (T_m), hairpins and primer-dimers using software available at www.idtdna.com. In addition, the primers were

designed to include an EcoR1 restriction site at their 5' end to facilitate cloning for subsequent DNA sequencing. A 4-base pair GCGC cap was also added to the primer to improve the efficiency of EcoR1 restriction enzyme digestion. All primers (desalted) were purchased from Integrated DNA Technologies (Coralville, IA, USA).

2.2.6 Reverse Transcription and Polymerase Chain Reaction (RT-PCR)

Reverse transcription was performed to convert mRNA to cDNA following the procedures for the first strand cDNA synthesis. All DNase, RT and PCR reagents were purchased from Invitrogen Life Technologies (Coralville, IA, USA).

Total RNA was treated with DNase to remove any contaminating DNA and ensure that only RNA was being amplified. Each 200 µL reaction tube contained 1 µg of total RNA, 250 ng of random primer, 1 µL of 10 mM dNTP mix, 4 µL of 5X first strand buffer, 2 µL of 0.1 M DTT, 1 µL of RNaseOUT and 1 µL of SuperScript RNase H-reverse transcriptase (250 U/µL) and sterile water to a final volume of 20 µL. RT was performed in a PTC-1000 programmable thermal controller (MJ Research Inc., Reno, NV, USA). RT reaction tubes were separately prepared for each bacterial gene: *E. coli* O157:H7 *rfbE*, *E. coli* O157:H7 *fliC*, *V. cholerae* O1 *rfbE* and *S.* Typhi *tyv*. Two controls were also prepared: a negative control containing all the reagents except the RNA template, and a DNase control for each bacterium containing the RNA template and all reagents except SuperScript RNase H-reverse transcriptase.

PCR was performed to test the specificity of all primer pairs prior to using them in a MPCR assay. Each 200 µL PCR tube contained 32 µL of sterile water, 2 µL of 10 mM dNTP mix, 1 µL of 50 mM $MgSO_4$, 5 µL of 10X amplification buffer, 1 µL of 10 µM forward primer, 1 µL of 10 µM reverse primer and 1 µL of (2.5 U/µL) Platinum

Pfx DNA polymerase. Four PCR reaction tubes were prepared: two for *E. coli* O157:H7, each containing 5 μL of O157:H7 RT as the cDNA template; one for *V. cholerae* O1, containing 5 μL of *V. cholerae* O1 RT as the cDNA template; and one for *S.* Typhi, containing 5 μL of *S.* Typhi RT as the cDNA template. Six controls were included: a RT negative control, containing all the PCR reagents, including 5 μL of the RT negative as template; a PCR negative control, containing PCR reagents and no template; and a DNase control for each gene containing all the PCR reagents and 5 μL of the RT DNase control as template. PCR was performed in an Applied Biosystems GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA) using an initial denaturation of 3 min at 94°C, amplification for 45 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s and extension at 72°C for 30 s, followed by a final extension at 72°C for 10 min.

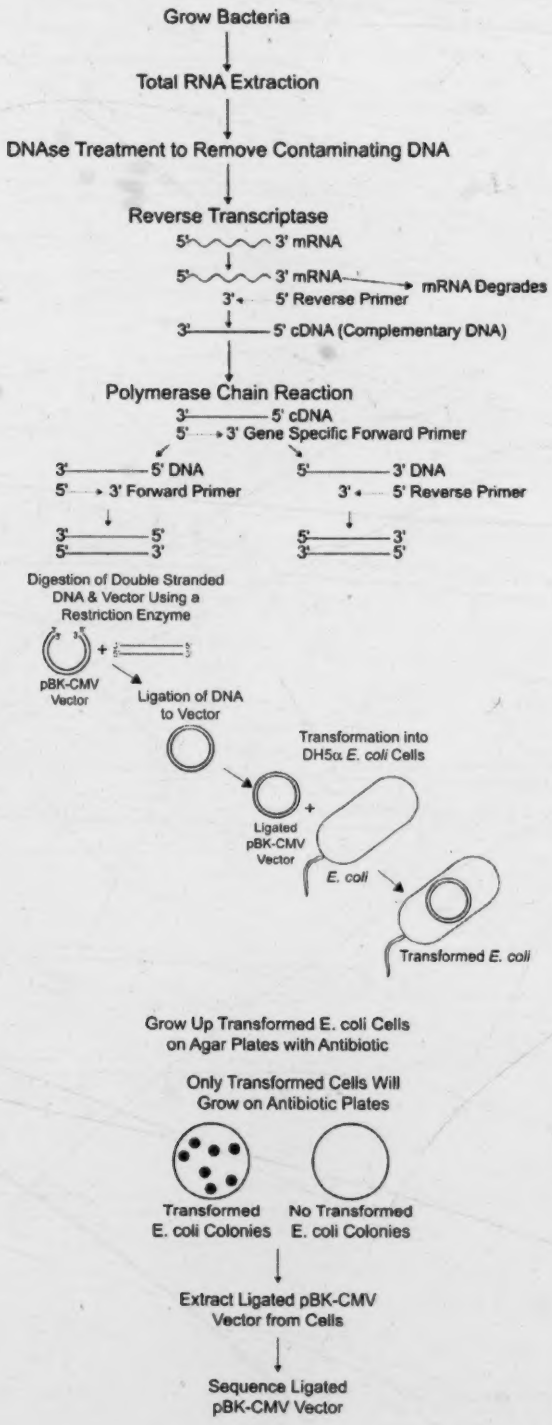
2.2.7 Cloning and DNA Sequencing

The four gene sequences were individually cloned into the EcoR1 site of a pBK-CMV vector and sequenced to confirm the specificity of the PCR primer pairs.

The protocol for cloning is shown in Figure 2.2. First, the PCR products were purified according to the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). Purified PCR product and pBk-CMV vector were digested using EcoR1 enzyme and react 3 buffer (Invitrogen Life Technologies, Carlsbad, CA, USA). Digestion was performed to produce compatible ends for ligation. pBk-CMV vector was further digested using alkaline phosphatase (USB Corporation, Cleveland, OH, USA) protocol to remove the 5'phosphoryl group. This added step prevented the vector from re-ligating without the PCR product. T4 DNA ligase (Invitrogen Life Technologies, Carlsbad, CA, USA) ligated the digested pBk-CMV vector and PCR product together as one piece. A

negative control was included consisting only of digested vector without the PCR product. Following a 2 h incubation at 23°C, the ligation mixture (all 20 μL) was used to transform DH5α chemically competent *E. coli* cells (Invitrogen Life Technologies, Carlsbad, CA, USA). Successfully transformed cells acquired kanamycin antibiotic resistance and therefore were screened on agar plates containing 30 μg/mL of kanamycin antibiotic (Invitrogen Life Technologies, Carlsbad, CA, USA). Half of the transformation mixture (100 μL) was streaked onto the plates and incubated at 37°C overnight. The following morning, a single colony from each plate was inoculated into 2 mL of LB broth containing 2 μL of kanamycin antibiotic and grown overnight in a 37°C incubator shaking at 125 rpm. All of the overnight culture (2 mL) was pelleted at 3000 rpm for 1 min in a micromax RF centrifuge (Thermo IEC, Needham Heights, MA, USA). The successfully transformed cells were purified according to the QIAprep miniprep purification kit recommended protocol (Qiagen, Valencia, CA, USA). The transformed vector, along with its gene-specific forward and reverse primer pair, was sent for sequencing to the DNA Services Laboratory, Department of Biochemistry, University of Alberta. Each sequenced gene was aligned with its corresponding GenBank accession number using a free alignment program available online at www.justbio.com.

Figure 2.2 Protocol for cloning and sequencing a bacterial gene sequence


Grow Bacteria
Total RNA Extraction
DNAse Treatment to Remove Contaminating DNA
Reverse Transcriptase
5' 3' mRNA
5' 3' mRNA
mRNA Degrades
3' 5' Reverse Primer
3' 5' cDNA (Complementary DNA)
Polymerase Chain Reaction
3' 5' cDNA
5' 3' Gene Specific Forward Primer
3' 5' DNA
5' 3' Forward Primer
5' 3' DNA
3' 5' Reverse Primer
3' 5'
5' 3'
5' 3'
3' 5'
Digestion of Double Stranded DNA & Vector Using a Restriction Enzyme
pBK-CMV Vector
Ligation of DNA to Vector
Transformation into DH5α E. coli Cells
Ligated pBK-CMV Vector
E. coli
Transformed E. coli
Grow Up Transformed E. coli Cells on Agar Plates with Antibiotic
Only Transformed Cells Will Grow on Antibiotic Plates
Transformed E. coli Colonies
No Transformed E. coli Colonies
Extract Ligated pBK-CMV Vector from Cells
Sequence Ligated pBK-CMV Vector

2.2.8 Multiplex Polymerase Chain Reaction

MPCR master mix, containing all the reagents except primer, was purchased from Qiagen (Valencia, CA, USA). MPCR procedures were adapted from the accompanying Qiagen multiplex PCR handbook. Primers were diluted to 2 μM for *E. coli* O157:H7 *rfbE*, 2 μM for *V. cholerae* O1 *rfbE*, 2 μM for *S.* Typhi *tyv* and 4 μM for *E. coli* O157:H7 *fliC* genes in a 10X TE mixture (10 mM Tris HCl, 1 mM EDTA, pH 8.0). A MPCR reaction mixture was prepared containing 25 μL of Qiagen multiplex PCR master mix, 5 μL of 10X TE primer mix, 5 μL from *E. coli* O157:H7 RT, 5 μL from *V. cholerae* O1 RT, and 5 μL from *S.* Typhi RT and sterile water to a final volume of 50 μL. Three controls were included to demonstrate that the assay was contamination-free. The first control contained PCR reagents and 5 μL from the RT negative control as template. The second control contained only the PCR reagents. The third control contained all the PCR reagents and 5 μL of each RT DNase control as template. MPCR was performed in an Applied Biosystems GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). PCR conditions consisted of an initial denaturation of 15 min at 94°C, amplification for 45 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 90 s and extension at 72°C for 90 s, followed by a final extension at 72°C for 10 min. The MPCR protocol took 3.5 hours to complete.

2.2.9 Agarose Gel Electrophoresis

Amplification products (5 μL) were separated and characterised on a 1.5% (w/v) agarose gel containing 0.5 μg/mL ethidium bromide. Ultra Pure™ agarose was purchased from Invitrogen Life Technologies (Carlsbad, CA, USA). 1X TAE electrophoresis buffer was prepared from a 50X TAE stock (242 g Tris Base, 57 mL acetic acid and 100 mL of

0.5 M EDTA, pH 8.0) (Fisher Scientific, Nepean, ON, Canada). DNA size markers were included in each gel to determine the molecular size of the PCR products. All products were visualised on a Syngene UV illuminator (Frederick, MD, USA).

2.3 Results and Discussion

2.3.1 Selection of the Target Genes

Three specific lipopolysaccharide genes and one flagellum gene were chosen as targets for detection. The *rfbE* gene of *E. coli* O157, the *rfbE* of *V. cholerae* O1, and the *tyv* gene of *S.* Typhi transcribe O-antigens. The surface O-antigen plays an important role in the infectivity of the pathogens [13]. For example, the wildtype *V. cholerae* O1 is at least 100-fold more pathogenic than its O-antigen mutants, as measured by LD_{50} [117]. The *rfbE* gene of *E. coli* O157 encodes the O157 lipopolysaccharide and is therefore unique to the *E. coli* O157 serogroup [118]. This gene has also been identified as a good viability marker because it is transcribed in all growth phases, from early exponential to late stationary phase [55]. The *rfbE* mRNA is only present in live cells and degrades quickly after the cell death [55].

The *fliC* gene of *E. coli* O157:H7 encodes the H7 flagellum, specifically indicating the presence of a functional flagellum [119]. *E. coli* O157 without a functional flagellum (designated as *E. coli* O157:H^-) has been suggested to be less pathogenic because it lacks motility [120]. Detection of both *rfbE* and *fliC* enables us to distinguish *E. coli* O157:H7 (presence of both *rfbE* and *fliC*) from *E. coli* O157:H^- (absence of *fliC*).

2.3.2 Primer Design

Initially, each primer pair was designed with similar T_m (within a few degrees) to ensure that the same annealing temperature could be used in the MPCR assay for all three

bacteria. Unfortunately, due to primer-dimer and hairpin interactions, they had to be redesigned. Subsequently, the melting temperatures were no longer within a narrow range, ranging instead from 58-68°C. Despite this limitation, under optimised conditions I achieved a similar efficiency for the MPCR assay using each primer pair.

Care was also taken to design the primers so that the amplified PCR products were different sizes, allowing them to be easily differentiated on an agarose gel following electrophoresis.

2.3.3 RT-PCR for Evaluation of Primers

Each primer pair was evaluated separately for specificity and functionality using traditional RT-PCR (one primer pair/one tube). The annealing temperature is one of the most important PCR parameters that ensures the fidelity of primers to the target sequence [121]. Because the T_m of all eight primers was greater than 60°C (except *E. coli* O157:H7 *rfbE* forward and reverse), this was chosen as the initial annealing temperature. Figure 2.3 shows that all four target genes are successfully amplified. After comparison with the DNA ladder in lane 1, the sizes of the amplified fragments were identified. They include a 419 base pair region of the *V. cholerae* O1 *rfbE* gene, a 319 base pair region of the *S.* Typhi *tyv* gene, a 239 base pair region of the *E. coli* O157:H7 *rfbE* gene and a 179 base pair region of the *E. coli* O157:H7 *fliC* gene.

Figure 2.3 RT-PCR of the *rfbE* gene of *V. cholerae* O1, the *tyv* gene of *S.* Typhi, and the *rfbE* and *fliC* genes of *E. coli* O157:H7

From the left:
Lane 1 – DNA ladder
Lane 2 – Negative control PCR reagents with no template
Lane 3 – Negative control RT and PCR reagents with no template
Lane 4 – 419 bp region of the *rfbE* gene of *V. cholerae* O1
Lane 5 – 329 bp region of the *tyv* gene of *S.* Typhi
Lane 6 – 239 bp region of the *rfbE* gene of *E. coli* O157:H7
Lane 7 – 179 bp region of the *fliC* gene of *E. coli* O157:H7

To ensure specificity, PCR primers need to be unique to a single region of the targeted gene. Initially, the sequence similarity of all primers used in this assay was evaluated against all the known sequences in the nucleic acid database GenBank. However, this process could not guarantee that the primers were unique because it is only a comparison against available sequences [50]. Therefore, the specificity of all primer pairs was further evaluated by sequencing each PCR product. Sequencing results confirmed that each primer pair successfully amplified the intended gene sequence.

2.3.4 RT-MPCR

Primers used in traditional PCR do not necessarily yield the same results in a MPCR assay because of changes in the concentrations and ratios of the reagents and

primers as well as potential interactions between the primer pairs [104]. In MPCR, templates from different bacteria put an additional demand on the assay requiring the conditions to be re-optimised [121]. As a result, primer pairs were also evaluated for specificity and functionality in the MPCR assay. According to the Qiagen MPCR manufacturer recommendations, primer pairs were initially prepared at a concentration of 2 μM. As a starting point, a 60°C annealing temperature and a 1 μg template concentration (1 μg of total RNA from each bacterium) was used. Following agarose gel electrophoresis, only three genes, the *rfbE* gene of *E. coli* O157:H7, the *rfbE* gene of *V. cholerae* O1 and the *tyv* gene of *S.* Typhi, were amplified. There was no amplification of the *fliC* gene of *E. coli* O157:H7. Several potential causes were identified, including the primer annealing temperature, the template concentration and the *fliC* primer pair ratio. Because the single PCR reaction successfully amplified the *fliC* gene at an annealing temperature of 60°C, temperature was not further investigated. Next, increasing concentrations of *E. coli* O157:H7 templates were evaluated. A range of template concentrations from 1 μg increasing to 10 μg (1, 3, 5, 7, 10 μg) failed to amplify the *fliC* gene.

Finally, the concentration of the *fliC* primer pair was explored. It was investigated last because the possibility of primer-dimer interactions interfering with the MPCR assay increases with higher primer concentrations. Following several attempts, an optimum primer ratio of 4 μM for the *E. coli* O157:H7 *fliC* gene, 2 μM for the *E. coli* O157:H7 *rfbE*, 2 μM for the *V. cholerae* O1 *rfbE* and 2 μM for the *S.* Typhi *tyv* genes was adopted. This ratio of primer pairs was necessary to yield similar intensities of all the PCR

products. It has been documented that the amplification of DNA varies with the primer concentration even when the template concentration remains constant [121].

As shown in Figure 2.4, the four bands corresponding to the targets were clearly detected at the optimised conditions. This gel also demonstrates that the four sets of primers maintained their specificity in the MPCR assay and can be applied for the simultaneous detection of *E. coli* O157:H7, *V. cholerae* O1 and *S.* Typhi.

Figure 2.4 Multiplex PCR of the *rfbE* gene of *V. cholerae* O1, the *tyv* gene of *S.* Typhi, and the *rfbE* and *fliC* genes of *E. coli* O157:H7

From the left:
Lane 1 – DNA ladder
Lane 2 – Hind III marker
Lane 3 – Negative control RT and PCR reagents with no template
Lane 4 – Negative control PCR reagents with no template
Lane 5 – Multiplex PCR *V. cholerae rfbE* 419 bp, *S.* Typhi *tyv* 329 bp, and *E. coli* O157:H7 *rfbE* 239 bp and *fliC* 179 bp

2.3.5 Specificity

The specificity of the RT-MPCR assay was confirmed using seven blinded RNA samples. All RNA samples were prepared in a blinded manner using previously extracted frozen RNA. These samples were randomly assigned a number from 1 to 7. RT-MPCR was performed for each RNA sample. By comparing the DNA bands in each sample with the DNA ladder in lane 1, all samples were correctly identified to contain every combination of these three bacteria, as shown in Figure 2.5. The identification of the seven samples was 100% correct. This demonstrates the potential application of my assay to accurately identify all four genes in an unknown sample.

Figure 2.5 Validation of the RT-MPCR assay using seven blinded RNA samples

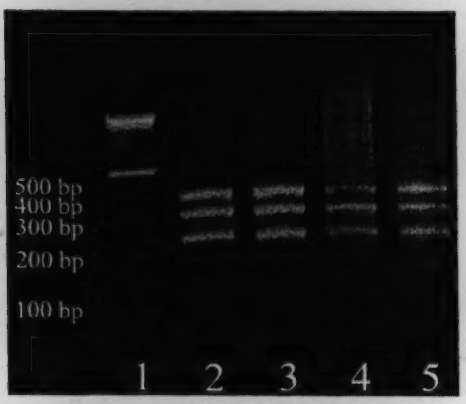
From the left:
Lane 1 – DNA ladder
Lane 2 – MPCR negative control
Lane 3 – RT negative control
Lane 4 – Blinded sample #1 *V. cholerae rfbE* gene
Lane 5 – Blinded sample #2 *E. coli* O157:H7 *rfbE* and *fliC* genes
Lane 6 – Blinded sample #3 *S.* Typhi *tyv* gene
Lane 7 – Blinded sample #4 *V. cholerae rfbE* and *E. coli* O157:H7 *rfbE* and *fliC* genes
Lane 8 – Blinded sample #5 *V. cholerae rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes
Lane 9 – Blinded sample #6 *V. cholerae rfbE and S.* Typhi *tyv* gene
Lane 10 – Blinded sample #7 *S.* Typhi *tyv* gene and *E. coli* O157:H7 *rfbE* and *fliC* genes
Lane 11 – 100 base pair ladder

2.3.6 Detection limit

The detection limit of the RT-MPCR assay was defined as the minimum concentration or cell number that all four bacteria genes were visible following agarose gel electrophoresis. Four different concentrations of template RNA were evaluated: 100 nM, 50 nM, 20 nM and 5 nM. For example, the first tube contained 100 nM each of *E. coli* O157:H7, *V. cholerae* O1 and *S.* Typhi RNA. Following reverse transcription, all the resulting cDNA (20 μL volumes) was added to the MPCR reagents. Since the entire

volume of RT was used, there was no dilution of the cDNA and the detection limit could easily be determined. All four genes were clearly visible at 100 nM and 50 nM, as shown in Figure 2.6 (Lanes 2 and 3). Although *rfbE* of *E. coli* O157:H7, *rfbE* of *V. cholerae* O1 and *tyv* of *S.* Typhi can be detected at concentrations as low as 5 nM RNA, the *fliC* gene of *E. coli* O157:H7 was not visible below 50 nM RNA (Figure 6, Lanes 4 and 5). Thus, the minimum initial RNA concentration required to confirm the presence of all three pathogens was determined to be 50 nM. .

Figure 2.6 Detection limit of the MPCR assay using RNA from *E. coli* O157:H7, *V. cholerae* O1 and *S.* Typhi



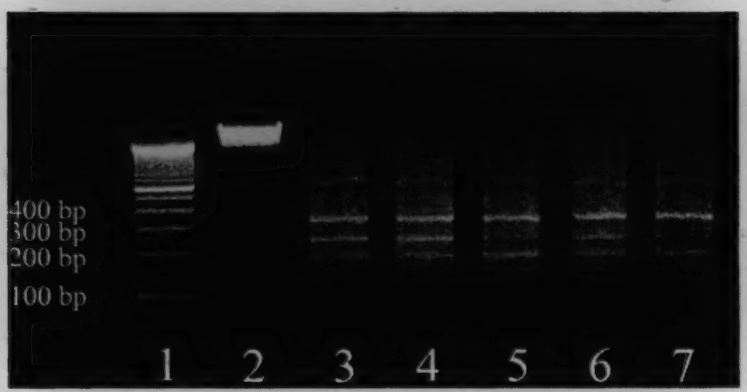
From the left:
Lane 1 – DNA ladder
Lane 2 – 100 nM
Lane 3 – 50 nM
Lane 4 – 20 nM
Lane 5 – 5 nM

2.3.7 Analysis of Clinical Isolates

Further application of the RT-MPCR assay was demonstrated by analysis of a number of bacteria obtained from patient stool samples. These bacteria were mixed in two groups to further test whether other selected bacteria interfere with the detection and identification of the target *E. coli* O157:H7 and *S.* Typhi. No clinical sample of *V. cholerae* O1 was available for inclusion in this test. Group 1 mixture contained five bacteria: *E. coli* O157:H7, *S.* Typhi, *L. monocytogenes*, *Y. enterocolitica* and a non-pathogenic *E. coli* strain. Group 2 mixture contained only non-pathogenic *E. coli*, *L.*

monocytogenes and *Y. enterocolitica*. Samples containing various numbers of bacterial cells from these two groups were prepared through serial dilution. For example, the first dilution contained 75000 cells of each of the five bacteria whereas the last dilution contained 30 cells of each of the five bacteria. The RNA from these samples was extracted, followed by RT-MPCR. The products were analysed by gel electrophoresis. Figure 2.7 shows the results from the samples containing 30-75000 cells of the group 1 mixture of bacteria. It clearly shows the target bands of 319 bp, 239 bp and 179 bp, corresponding to the *tyv* gene of *S.* Typhi, and the *rfbE* and *fliC* genes of *E. coli* O157:H7, respectively. This demonstrates that the RT-MPCR specifically detects the target genes and that there is no interference from the other tested bacteria present at the same concentration.

Figure 2.7 Agarose gel electrophoresis of group 1 samples with the dilutions containing equal cell numbers of each bacteria species



From the left:
Lane 1 – DNA ladder
Lane 2 – Hind III marker
Lane 3 – 75000 cells
Lane 4 – 7500 cells
Lane 5 – 1500 cells
Lane 6 – 150 cells
Lane 7 – 30 cells

No band was observed in seven samples containing 30-75000 cells of non-pathogenic *E. coli*, *L. monocytogenes* and *Y. enterocolitica*. Thus, there were no false positive results from bacterial samples that did not contain the target genes.

The detection limit of the assay was evaluated using the serially diluted clinical samples. The *rfbE* and *fliC* genes of *E. coli* O157:H7 and the *tyv* gene of *S.* Typhi are visible in all dilutions from 30-75000 cells (also shown in Figure 2.7). In the last dilution (approximately 30 cells), the *tyv* gene of *S.* Typhi is clearly visible, whereas it is becoming increasingly difficult to visualize the *rfbE* and *fliC* genes of *E. coli* O157:H7. Therefore, the detection limit of the MPCR assay was determined to be 30 cells for the simultaneous detection of *S.* Typhi and *E. coli* O157:H7. These results are in agreement with other published methods reporting a PCR detection limit for most bacterial species between 4-100 cells [60]. As well, the detection limit is lower than the estimated oral infective dose of *S.* Typhi at 10^4 cells [122].

2.3.8 Dead Cell Control

In order to confirm that the RT-MPCR assay detects mRNA and that DNA does not cause a false positive result, a dead cell control was included. The sample contained approximately 10^7 *E. coli* O157:H7 cells that were boiled for 10 min in order to kill all the bacterial cells. The same procedures as described above for the analysis of bacterial mixtures, including extraction of total RNA and RT-MPCR, were carried out on the dead cell control. Following agarose gel electrophoresis of the products, no band was visible, suggesting the absence of target RNA in the dead cell control following boiling inactivation. This demonstrates that the RT-MPCR assay specifically detects *E. coli* O157:H7 mRNA.

2.4 Conclusion

An assay combining RT with single-tube MPCR was successfully developed and validated for simultaneous detection of viable *E. coli* O157:H7, *V. cholerae* O1 and *S.*

Typhi. Analysis of five clinical isolates showed that the assay was able to detect and accurately identify as few as 30 cells of *E. coli* O157:H7 and *S.* Typhi and that the presence of other selected bacterial species did not interfere with the analysis. The results demonstrate the specificity and detection limit of this assay. No sample of *V. cholerae* O1 was available for inclusion in the clinical isolates and therefore no statement can be made regarding its detection limit or the interference of other selected bacteria on its detection.

Chapter 3: DNA Microarray Detection of *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv*, *E. coli* O157:H7 *rfbE* and *fliC* genes

3.1 Introduction

Most DNA microarrays have been developed for gene expression and single nucleotide polymorphisms (SNPs) studies in human genome research. This technology is extremely useful because it allows for rapid, flexible and simultaneous detection of hundreds to thousands of genes. In addition, DNA microarray technology is advantageous because it offers a lower detection limit compared to conventional methods such as gel electrophoresis. With gel electrophoresis, the detection limit is determined by the ability of ethidium bromide to intercalate the DNA bands and fluoresce under UV light; therefore it does not have a good resolution. As a result, false negative results can occur when the DNA concentration is too low to be visible on a gel [73].

Given the advantages of microarrays, it is surprising that there have been only a small number of arrays developed for the detection of bacterial pathogens. To date, arrays have been developed for the *stx1*, *stx*2, *eae*, *fliC*, *rfbE* and *tir* virulence genes of *E. coli* O157:H7 [45, 73, 123], the *gyrB* gene of *E. coli*, *Shigella flexneri*, *Shigella sonnei,* and *S.* Typhi and Paratyphi [124], the 16S rRNA gene of *Listeria monocytogenes* [95] and the *gyrB* gene of *Mycobacterium* species [125]. Unfortunately, none of the previously developed arrays have combined RT- PCR amplification with DNA microarray detection and, as a result, use DNA in the sample instead of mRNA as the starting template. Therefore, they are not useful for detecting viable bacteria in clinical and water samples. Also, only one of the developed methods detects the *rfbE* gene that has been identified as a good viability marker in *E. coli* O157:H7 [123]. To date, no published microarray

techniques have been developed to simultaneously detect viable *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1 based on their O antigen genes. To detect viable bacteria, the RT-PCR assay, developed in Chapter 2, section 2.2.6, was combined with DNA microarray detection.

Specific capture and reporters were designed for the *rfbE* and *fliC* genes of *E. coli* O157:H7, the *rfbE* gene of *V. cholerae* O1 and the *tyv* gene of *S.* Typhi. These genes were chosen as targets because they have previously been identified to be specific, as discussed in section 2.3.5. DNA microarray addressing parameters, including capture addressing time, target addressing time and voltage, were evaluated to achieve the highest signal-to-background ratio (SBR) with the shortest addressing time. Once the assay was established, the relationship between target concentration and the SBR was determined using various concentrations. Finally, the DNA microarray detection technique was examined for its detection limit and specificity and was compared to agarose gel electrophoresis using RT-PCR products.

3.2 Materials and Methods

3.2.1 Capture and Reporter Design

Captures and reporters were designed for the *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes based on the previously obtained GenBank sequence data, as shown in Figure 3.1. In order to achieve optimum capture/target and target/reporter hybridisation on the DNA microarray, several factors were taken into consideration, including capture length, GC content, T_m and secondary structure. Captures were designed to be short in order to diminish secondary structure, compared to longer captures [126]. Secondary structure was further minimised by designing captures

to contain less than 50% GC nucleotide content. Captures were also designed to have a melting temperature (T_m) greater than 60°C to ensure that they remained hybridised to the targets while thermal stringency was applied. The recommended T_m for the reporters is in the range of 28-40°C, lower than the T_m of the captures and, as a result, mismatch reporters are easily removed, allowing the differentiation between perfectly matched and mismatched target/reporter hybrids [127]. The capture and reporter oligonucleotides were evaluated for T_m, GC content, primer-dimers and hairpin structures using software available from the Integrated DNA Technologies website, at www.idtdna.com.

Figure 3.1 Capture and reporter design for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes based on GenBank data

a) *V. cholerae* O1 *rfbE* gene (Accession # X59554)

Complementary capture sequence highlighted in yellow
Complementary reporter sequence highlighted in pink

Gene Sequence 5'

5641

```
tatattgatc gcttcgaaac tgagtttgcg gagtttttaa aagtaaagca cgccacaaca

gtatctaatg gaacagttgc gctacatttg gcaatgagcg cgttgggaat aactcaaggc
                                Capture 3' gcaacccttа ttgagttccg
gatgaagtga ttgtacc[redacted]gcctcgg ttaataccat agtccagtgt
ctacttcact aacatggttg taagtgaata caa Reporter 5'
ggtgcgttac ccgtttttgc tgaaatcgaa ggtgagtctc tacaagtgag cgtagaggac

gttaaacgta aaataaataa aaagacaaaa gcagttatgg ccgttcacat atatggacaa

gcttgcgata ttcaatcttt aagagatctg tgtgatgagc acggcctcta tttaattgaa

gactgtgcag aagcaatagg tactgctgtt aatggcaaaa aagtaggtac atttggcgat 6060
```

b) *S.* Typhi *tyv* gene (Accession # M29682)

Complementary capture sequence highlighted in yellow
Complementary reporter sequence highlighted in pink

Gene Sequence 5'

1201

```
agcttttaat taccggtgga tgtggcttcc ttgggagtaa tcttgcctcc tttgctttaa

gtcaagggat tgatttaatt gtattcgata atctatcacg taaaggtgca acagataatt

tacattggtt atcctcctta ggaaactttg agtttgtaca tggtgat attcgcaacaaaa
                                              Capture 3' taagcgttgtttt
atgatgttac aagattaata [redacted]atag ctgttttcat cttgcaggtc
tactacaatg ttctaatt   tgattcatat acggac Reporter 5'
aagtggcaat gactacatct attgacaatc cttgtatgga ttttgaaatt aatgtaggtg

gaactttaaa tttacttgag gcagtacggc 1530
```

c) *E. coli* O157:H7 *rfbE* gene (Accession # AE005429)

Complementary capture sequence highlighted in yellow
Complementary reporter sequence highlighted in pink

Gene Sequence 5'

7441

```
gtgcttttga tatttttccg agtacattgg catcgtgtgg acagggtaaa aaactggcct

tgtttcgatg agtttatctg caaggtgatt ccttaattcc tctctttcct ctgcggtcct
                                                    Capture 3' gga
agttagaatt gagaccat[redacted]aacatct ttactttcct tgtggacttg
tcaatcttaa ctctggtagg ttattcacac ttt Reporter 5'
tacaagactg ttgatatttt ttttataaat atcagcaatt tcacgttttc gtgatataaa 7680
```

d) *E. coli* O157:H7 *fliC* gene (Accession # AE005415.1)

Complementary capture sequence highlighted in yellow
Complementary reporter sequence highlighted in pink

Gene Sequence 5'

1981

```
gttggtcgtt gcagaaccag cactggtagt ctcagtagtg attttacccg cggagttcac

atttacagca gtaccaattg ctggagcata atctttattg gtatcagtcg ctgaagcata
          Capture 3' gacctcgtat tagaaataac catagtcagc gacttcgtat
cccggc[redacted]c cgttatcctt gttaacgctg taagagactg tatagtcggc 2160
      ttgt cagtgtctc Reporter 5'
```

Although each capture and reporter were designed to be specific for their complementary target, it was impossible to design them to have the same number of nucleotides, the same GC content or the same T_m because of the similarity between the *rfbE* genes of *V. cholerae* O1 and *E. coli* O157:H7 and the *tyv* gene of *S.* Typhi. In order to immobilise the captures to the streptavidin in the hydrogel permeation layer covering the cartridge, a biotin molecule was included at the 3' end. The capture and reporter designs are shown in Table 3.1.

Table 3.1 Capture and reporter design for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes

Target Bacteria	Target Gene and Accession Number	Gene Sequence Position 5' ⇒ 3' and Size	Capture Design 5' ⇒ 3' Labeled with Biotin at 3' end Size GC content T_m	Reporter Design 5' ⇒ 3' Labeled with Cy^3 at 5'end Size T_m
V. cholerae O1	*rfbE* gene (Accession # X59554)	5641-6060 419 bp	ggtacaatcacttcatcgccttga gttattcccaacg 37 bp 17 GC T_m = 67 °C	aacataagtgaatgtt 16 bp T_m = 39 °C
S. Typhi	*tyv* gene (Accession #M29682)	1201-1530 329 bp	ttaatcttgtaacatcatttttgttgg aat 31 bp 7 GC T_m = 57 °C	caggcatatacttagt 16 bp T_m = 41 °C
E. coli O157:H7	*rfbE* gene (Accession # AE005429)	7441-7680 239 bp	atggtctcaattctaactagg 21 bp 8 GC T_m = 50 °C	tttcacacttattgg 15 bp T_m = 39 °C
E. coli O157:H7	*fliC* gene (Accession # AE005415.1)	1981-2160 179bp	tatgcttcagcgactgataccaat aaagattatgctccag 40 bp 11 GC T_m = 65 °C	ctctgtgactgtt 13 bp T_m = 38 °C

3.2.2 Sample Preparation

Based on the designs, captures and reporters were synthesised and purified (using HPLC) at Integrated DNA Technologies (Coralville, IA, USA). Once received, each was diluted in 200 µL of sterile water (that had been autoclaved) and immediately frozen in a 20°C freezer until further use. Histidine buffer was prepared at two concentrations: 50 mM (with an optimum conductivity <100 µS/cm) and 100 mM (with an optimum conductivity 100-200 µS/cm). The 50 mM buffer was stored in a 4°C fridge for up to 1 week whereas the 100 mM buffer was stored in a -20°C freezer for up to 2 months. Since the 50 mM histidine buffer was not stable, its conductivity was tested using a Horiba twin compact B-173 conductivity meter (Horiba Ltd.,Irvine, CA, USA) prior to each use. Low salt buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4) and high salt buffer (50 mM NaH_2PO_4/Na_2HPO_4, pH 7.4, 500 mM NaCl) were prepared monthly. These reagents were purchased in powder form from Fisher Scientific (Nepean, ON, Canada).

Targets were produced using conventional RT-PCR as previously discussed in section 2.2.6. Briefly, total RNA was extracted from *V. cholerae* O1, *S.* Typhi and *E. coli* O157:H7. Reverse transcription was performed separately for each gene using a random primer. Five microlitres of each RT preparation was added to each PCR tube containing all PCR reagents and one set of gene-specific primers. Successfully amplified gene products were purified according to the manufacturer's protocol using a QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). All products were quantified using a lambda Hind III marker (Invitrogen Life Technologies, Carlsbad, CA, USA) following agarose gel electrophoresis. PCR products (also called targets) were diluted to various concentrations in sterile water. Immediately prior to use, each DNA sample was

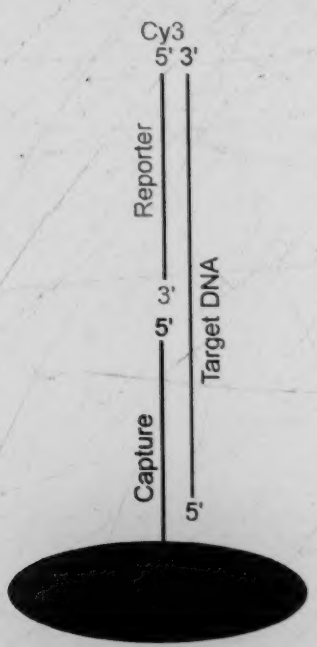
denatured to single strands by heating at 95°C for 5 min followed by a 3 min incubation on ice. The sample was diluted in 100 mM histidine buffer and sterile water to a total volume of 60 µL. According to Nanogen recommendations and previous work, captures were diluted to 0.5 µM in 100 mM histidine and water [128, 129]. A background control (50 mM histidine) was prepared by diluting 100 mM histidine with water. All target, capture and background controls were prepared in designated wells on a 96-well plate (Nalgene Inc., Rochester, NY, USA) to a final volume of 60 µL. The 60 µL solution from one well was sufficient to address 100 test sites on the DNA microarray.

3.2.3 Instrumentation

A NanoChip™ Workstation (Nanogen, San Diego, CA, USA) was used as a platform for this study. The NanoChip™ workstation consists of a NanoChip™ cartridge, a loader and a reader. Within the cartridge is a semiconductor device that consists of 100 individual test sites connected to an electrode laid out in a 2 mm x 2 mm grid. The cartridge acts as an interface providing the electrical and fluidic connections to the instrument. The semiconductor is coated with a hydrogel permeation layer containing streptavidin binding sites. Each test site is 80 microns in diameter and contains approximately 10^9 streptavidin molecules [130].

The principle of the DNA microarray assay involves three steps, immobilisation of capture (step 1), hybridisation with targets (step 2) and detection of reporter fluorescence (step 3). The resulting hybrid is shown in Figure 3.2.

Figure 3.2 Hybrid formed by capture and reporter binding to its complementary target



A list of DNA microarray terminology is listed in Table 3.2.

Table 3.2 Electronic microarray terminology

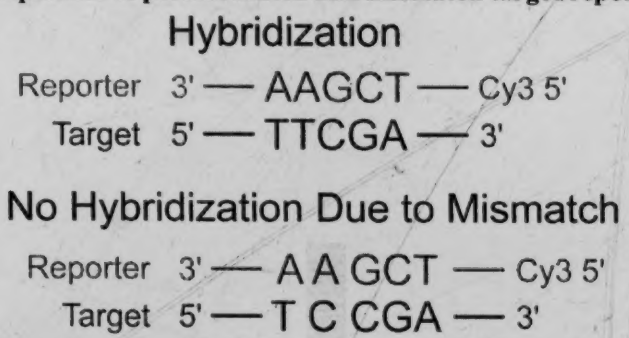
Oligonucleotide: A series of nucleotides (bases) in succession
Capture: A 3' biotinylated oligonucleotide that is complementary to the target gene sequence
Target: A gene sequence of the target bacterium
Reporter: A 5' fluorescently labeled oligonucleotide that hybridises with its complementary target
Addressing: Immobilising capture and complementary target to electronically activated test sites on the array
Reporting: Evaluating hybridisation between captures and targets by applying fluorescently labeled reporter to the array

The loader controls the addressing of both the capture and target (step 1 and 2). Within the loader, several addressing parameters can be controlled, including the location of the activated test sites, the addressing time and the addressing voltage. The reader

detects the fluorescence at each test site on the array (step 3) after fluorescently labelled reporter (Cy^3 dye molecule) complementary to the target is incubated on the array. Hybridisation is determined by the fluorescence intensity and the identification of the target gene sequence is based on its location on the array.

Two unique reader processes allow for the discrimination of mismatch hybrids: low salt washes and thermal stringency. Low salt washes create an unfavourable environment for hybridisation and will result in the removal of mismatch hybrids. These washes are automated and, as such, ensure quality control and consistency of washing between arrays. Thermal stringency allows the temperature of the array to be raised from room temperature to the T_m of the reporter. Elevated temperatures remove mismatched reporters because it becomes increasingly difficult for the reporter to remain bound at temperatures close to its T_m. The definition and consequence of a mismatch target/reporter hybrid is demonstrated in Figure 3.3.

Figure 3.3 Consequences of perfect match and mismatch target/reporter hybrids


Hybridization
Reporter 3' — AAGCT — Cy3 5'
Target 5' — TTCGA — 3'
No Hybridization Due to Mismatch
Reporter 3' — A A GCT — Cy3 5'
Target 5' — T C CGA — 3'

3.2.4 Instrument Calibration

The NanoChip™ Workstation was tested monthly to ensure that all parameters were functioning within specifications. Three test cartridges were used to evaluate its performance: continuity, temperature and fluorescence. The conductivity cartridge tested

all four loader positions and the reader position to ensure each test site electrode was conducting current within the specified range of 800-1200 nA. The temperature cartridge was tested in the reader to ensure the temperature was within (±) 2°C of the reported value. The fluorescence cartridge tested the low, medium and high gain for both the green and red laser used in the reader. The monthly tests yielded results within normal specifications.

3.2.5 Sample Loading

A map identifying the contents of each well and its corresponding test site on the microarray were created in the loader (called a loader map). An example of a typical loader map is shown in Figure 3.4.

Figure 3.4 Sample loading format from the well plate to the DNA microarray cartridge

Well Plate

	1	2	3	4	5
A	50 mM Histidine (background control)	*E. coli* O157:H7 *fliC* capture	*E. coli* O157:H7 *rfbE* capture	*V. cholerae O1* *rfbE* capture	*S.* Typhi *tyv* capture
B	*E. coli* O157:H7 *fliC* target	*E. coli* O157:H7 *rfbE* target	*V. cholerae* O1 *rfbE* target	*S.* Typhi *tyv* target	

Capture Loading Format

	1	2	3
A	50 mM Histidine		50 mM Histidine
B		50 mM Histidine	
C	*E. coli* O157:H7 *fliC* capture		*E. coli* O157:H7 *fliC* capture
D		*E. coli* O157:H7 *fliC* capture	
E	*E. coli* O157:H7 *rfbE* capture		*E. coli* O157:H7 *rfbE* capture
F		*E. coli* O157:H7 *rfbE* capture	
G	*V. cholerae* O1 *rfbE* capture		*V. cholerae* O1 *rfbE* capture
H		*V. cholerae* O1 *rfbE* capture	
I	*S.* Typhi *tyv* capture		*S.* Typhi *tyv* capture
J		*S.* Typhi *tyv* capture	

Target Loading Format

	1	2	3
A	No target addressed (background control)		No target addressed (background control)
B		No target addressed (background control)	
C	*E. coli* O157:H7 *fliC* target		*E. coli* O157:H7 *fliC* target
D		*E. coli* O157:H7 *fliC* target	
E	*E. coli*O157:H7 *rfbE* target		*E. coli* O157:H7 *rfbE* target
F		*E. coli* O157:H7 *rfbE* target	
G	*V. cholerae* O1 *rfbE* target		*V. cholerae* O1 *rfbE* target
H		*V. cholerae* O1 *rfbE* target	
I	*S.* Typhi *tyv* target		*S.* Typhi *tyv* target
J		*S.* Typhi *tyv* target	

Triplicate background samples (50 mM histidine) were included on each cartridge. Histidine buffer was used to monitor background fluorescence because the buffer promotes hybridisation. In the presence of histidine, a reporter could hybridise to a site in the absence of its complementary target. Also, triplicates of each capture site were included to account for variations in fluorescence intensity. Initially, each site was loaded in a triangle position to avoid any potential interference from adjacent test sites, as suggested by Gong et al. [129]. However, this loading format was found to be unnecessary and, as a result, all 100 test sites on each cartridge were used.

3.2.5.1 Loader Protocol

A loader protocol consists of seven steps that are described below:

Step 1: Design loader map
Step 2: Test conductivity of test sites
Step 3: Address biotinylated captures
Step 4: Remove unbound captures following cartridge rinse with 50 mM histidine
Step 5: Address targets
Step 6: Remove unbound captures following cartridge rinse with 50 mM histidine
Step 7: Remove cartridge and rinse with high salt buffer in preparation for reporter hybridisation

3.2.5.2 Investigation of Sample Addressing Parameters

It was necessary to investigate addressing parameters in order to achieve the highest SBR (a minimum of 5:1) and the shortest addressing time for a sample. Capture addressing time, target addressing time and target addressing voltage are all important addressing parameters that affect SBR. Initial addressing parameters were chosen based on previous work by Gong et al. [129]. Parameters were evaluated by changing only one parameter per experiment while holding the others constant. For example, capture addressing times were evaluated while target addressing time, voltage and concentration were kept constant. In this manner, each addressing parameter was independently evaluated of the others. For all these experiments, 15 nM target concentrations were used.

3.2.6 Reporting

Prior to use, gene-specific reporters were diluted to 1 μM in high salt buffer to a final volume of 100 μL. The reporter solution was applied to the cartridge and allowed to hybridise to its corresponding target. After 3 min, the solution was removed by pipette and replaced with 100 μL of high salt buffer. The high salt buffer in the cartridge was necessary to allow the reader optics to be focused on the cartridge. The camera system was adjusted to assign coordinate points to the test sites by locating each corner of the cartridge. Only test sites that had been addressed were scanned, thereby decreasing the analysis time. The order of each function in the reader protocol with and without thermal stringency is described as follows.

Sample Reader Protocol without Thermal Stringency

Step 1: Adjust camera system to locate all four corners of the cartridge
Step 2: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 3: Rinse cartridge with low salt (500 µL at 75 µL/s)
Step 4: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 5: Rinse cartridge with low salt (500 µL at 75 µL/s)
Step 6: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 7: Remove cartridge and store in a sealed container at 4°C

Sample Reader Protocol with Thermal Stringency

Step 1: Adjust camera system to locate all four corners of the cartridge
Step 2: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 3: Rinse cartridge with low salt (500 µL at 75 µL/s)
Step 4: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 5: Rinse cartridge with low salt (500 µL at 75 µL/s)
Step 6: Raise cartridge temperature to 35°C
Step 7: Rinse cartridge with low salt (500 µL at 75 µL/s)
Step 8: Lower cartridge temperature to 23°C prior to scan
Step 9: Scan test sites (low gain, automatic accumulation, using green laser for Cy^3)
Step 10: Repeat Step 6-9 each time increasing the temperature by 1°C until 45°C
Step 11: Remove cartridge and store in a sealed container at 4°C

3.2.7 Data Analysis

Analysis of the fluorescence intensities on individual test sites was performed using built-in NanoChip™ configuration software. In the analysis, fluorescence intensities from the 50 mM histidine sites were used as the background. Fluorescence intensity values from the same capture loaded test sites were analysed for statistical agreement. The Q test was used to reject values that were not in agreement with the others in the set [131]. Fluorescence intensity values were averaged and divided by the background to obtain signal-to-background ratios.

$$SBR = \frac{Average\ Fluorescence\ Intensity}{Background}$$

3.2.8 Re-usability

The re-usability of the cartridge was evaluated by addressing and removing

E. coli O157:H7 *rfbE* and *S.* Typhi *tyv* targets. Beginning with a new cartridge, *rfbE* and *tyv* targets were addressed to their complementary capture test sites. Reporters for *rfbE* and *tyv* were applied to the cartridge and the fluorescence intensity was reported by the reader. The cartridge was reused by removing the hybridised targets and reporters following a 3 min incubation with 0.1 M NaOH solution. The NaOH solution was removed and the cartridge was rinsed three times with sterile water. The cartridge was filled with 100 μL of 50 mM histidine and was readdressed and reported using new *rfbE* and *tyv* targets. The criterion for acceptable re-usability of the assay was defined as a SBR greater than 5:1.

3.2.9 Detection Limit

The detection limit of the DNA microarray technique was compared to traditional agarose gel electrophoresis detection. Four concentrations of *E. coli* O157:H7 *rfbE* and *fliC* DNA were run on an agarose gel and on the DNA microarray: 100 nM, 15 nM, 1 nM and 0.25 nM. The detection limit was further compared using approximate cell numbers. RT-PCR was performed on group 1, clinical sample #4 (approximately 150 cells as discussed in section 2.3.7) using *rfbE* and *fliC* gene-specific primers. The resulting PCR product was diluted 10 times and 100 times equivalent to approximately 15 and 2 cells. The samples were analysed in parallel by agarose gel electrophoresis and the DNA microarray.

3.2.10 Specificity

Preliminary experiments were undertaken to determine the specificity of the DNA microarray technique. Fifteen nanomolar *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *fliC* targets were addressed individually to *E. coli* O157:H7 *fliC* capture test

sites. Then, *E. coli* O157:H7 *fliC* reporter was applied to the array and the SBR was reported following two low salt washes. The *E. coli* O157:H7 *fliC* capture and reporter were determined to be specific if the SBR from the test sites with the mismatch targets, *V. cholerae* O1 *rfbE* and *S.* Typhi *tyv*, were low compared to the complementary target *E. coli* O157:H7 *fliC*. No *E. coli* O157:H7 *rfbE* target was available for inclusion in this experiment.

3.3 Results and Discussion

3.3.1 Capture and Reporter Specificity

The specificity of each capture and reporter for its intended target was analysed against all published gene sequences in GenBank using a BLAST search. Only captures and reporters that were unique to the target genes and that did not display significant similarity with non-targeted gene sequences were accepted. Sequence alignments were performed to determine the similarity between the non-targeted gene sequences and the reverse strand of each capture as well as between the non-targeted gene sequences and the reverse strand of each reporter. The sequence similarity was determined by comparing the maximum number of consecutive identical nucleotides out of the total possible nucleotides. Minimal sequence similarity is important because mismatches hybridise more easily as the number of consecutive identical nucleotides increases.

A sample calculation (a) of the sequence similarity between the *E. coli* O157:H7 *fliC* gene sequence and the reverse strand of the *E. coli* O157:H7 *fliC* capture is included below. The sequence alignment in (a) shows that there are 40 consecutive identical nucleotides between the *E. coli* O157:H7 *fliC* gene sequence and the reverse strand of the *E. coli* O157:H7 *fliC* capture, thereby demonstrating capture specificity.

Sample calculation:

a) Sequence alignment of the *E. coli* O157:H7 *fliC* gene sequence (Line 1) and the reverse strand of the *E. coli* O157:H7 *fliC* capture (Line 2).
Note: a dash represents no nucleotide match and an asterisk represents a perfect nucleotide match

```
Line 1 GTTGGTCGTTGCAGAACCAGCACTGGTAGTCTCAGTAGTGATTTTACCCG
Line 2 -------------------------------------------------

Line 1 CGGAGTTCACATTTACAGCAGTACCAATTGCTGGAGCATAATCTTTATTG
Line 2 ------------------------------CTGGAGCATAATCTTTATTG
                                     ********************

Line 1 GTATCAGTCGCTGAAGCATACCCGGCAACAGTCACAGAGCCGTTATCCTT
Line 2 GTATCAGTCGCTGAAGCATA----------------------------
       ********************

Line 1 GTTAACGCTGTAAGAGACTGTATAGTCGGCAACGTTAGTGATACCACCTT
Line 2 -------------------------------------------------

Line 1 TATCATCCACATAAGACTTCGCAGCATCACTGGATTCACC
Line 2 ----------------------------------------
```

The next sample calculation (b) shows the sequence alignment between the *E. coli* O157:H7 *fliC* gene sequence and the reverse strand of the *V. cholerae* O1 *rfbE* capture. The sequence alignment shows that a maximum of 7/37 consecutive nucleotides in the *V. cholerae* O1 *rfbE* capture are identical to the *E. coli* O157:H7 *fliC* gene. Therefore, this sequence has little similarity with the *V. cholerae* O1 *rfbE* capture and should not hybridise.

Sample calculation:

b) Sequence alignment of the *E. coli* O157:H7 *fliC* gene sequence (Line 1) and the reverse strand of the *V. cholerae* O1 *rfbE* capture (Line 2).

```
Line 1 GTTGGTCGTTGCAGAACCAGCACTGGTAGTCTCAGTAGTGATTTTACCCG
Line 2 ------CGTTG-GGAATAACTCAAGGCGAT----GAAGTGATTGTACC--
             *****  ***  *     **   *    * ******* ****

Line 1 CGGAGTTCACATTTACAGCAGTACCAATTGCTGGAGCATAATCTTTATTG
Line 2 --------------------------------------------------

Line 1 GTATCAGTCGCTGAAGCATACCCGGCAACAGTCACAGAGCCGTTATCCTT
Line 2 --------------------------------------------------

Line 1 GTTAACGCTGTAAGAGACTGTATAGTCGGCAACGTTAGTGATACCACCTT
Line 2 --------------------------------------------------

Line 1 TATCATCCACATAAGACTTCGCAGCATCACTGGATTCACC
Line 2 -----------------------------------
```

Table 3.3 shows the results obtained for each capture with the *rfbE* and *fliC* genes of *E. coli* O157:H7, *the rfbE* gene of *V. cholerae* O1 and the *tyv* gene of *S.* Typhi following sequence alignment.

Table 3.3 Maximum numbers of consecutive identical nucleotides between gene sequences and the reverse capture strand

		Reverse Capture Strand Sequence			
		E. coli* O157:H7 *rfbE	***E. coli* O157:H7 *fliC***	***V. cholerae* O1 *rfbE***	***S.* Typhi *tyv***
Gene Sequence	***E. coli* O157:H7 *rfbE***	21/21	6/40	5/37	3/31
	E. coli* O157:H7 *fliC	4/21	40/40	7/37	3/31
	V. cholerae* O1 *rfbE	4/21	3/40	37/37	4/31
	S.* Typhi *tyv	5/21	6/40	6/37	31/31

Table 3.4 shows the results obtained for each reporter with the *rfbE* and *fliC* genes of *E. coli* O157:H7, the *rfbE* gene of *V. cholerae* O1 and the *tyv* gene of *S.* Typhi following sequence alignment.

Table 3.4 Maximum numbers of consecutive identical nucleotides between gene sequences and the reverse reporter strand

		Reverse Reporter Oligonucleotide Sequence			
		E. coli O157:H7 *rfbE*	*E. coli* O157:H7 *fliC*	*V. cholerae* O1 *rfbE*	*S.* Typhi *tyv*
Gene Sequence	*E. coli* O157:H7 *rfbE*	15/15	2/13	4/16	3/16
	E. coli O157:H7 *fliC*	3/15	13/13	3/16	3/16
	V. cholerae O1 *rfbE*	3/15	2/13	16/16	5/16
	S. Typhi *tyv*	3/15	2/13	4/16	16/16

3.3.2 Sample Addressing Parameters

As previously discussed, it was necessary to investigate the addressing parameters to maximise the efficiency of capture and target delivery to test sites. Factors affecting addressing efficiency include capture and reporter characteristics such as length, secondary structure and T_m as well as experimental parameters such as capture addressing time, target addressing time and target addressing voltage.

3.3.2.1 Capture Addressing Time

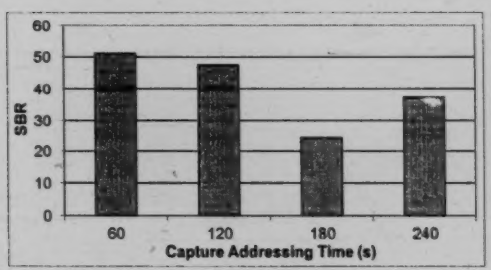
Within the loader, the capture addressing time can be adjusted from a minimum of 10 s to a maximum of 600 s. Although Nanogen recommends a 60 s capture addressing time, it was still necessary to optimise because it was hypothesised that capture addressing time would vary according to capture length. As a result, longer captures may require increased addressing times for streptavidin-biotin bonding compared to shorter ones.

As shown in Figure 3.5, SBRs were obtained using four different capture addressing times: 60, 120, 180 and 240 s. The SBR obtained ranged from 12-55 and were different for each capture studied. These data suggest that the highest SBR for all four captures is obtained by a 60 s capture addressing time. It is noteworthy that the captures

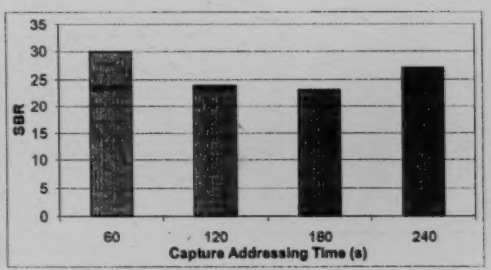
do not differ sufficiently in size (37 bp for the *V. cholerae* O1 *rfbE* capture, 31 bp for the *S.* Typhi *tyv* capture, 21 bp for the *E. coli* O157:H7 *rfbE* capture and 40 bp for *E. coli* O157:H7 *fliC* capture) to require different addressing times.

Figure 3.5 Influence of capture addressing time on SBR for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes

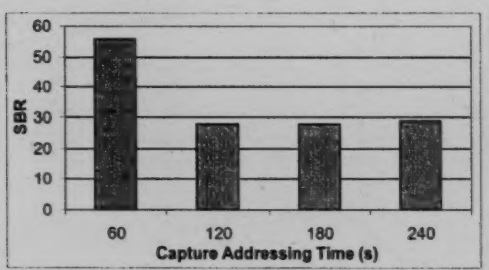
a) *V. cholerae* O1 *rfbE* capture



b) *S.* Typhi *tyv* capture



c) *E. coli* O157:H7 *rfbE* capture



d) *E. coli* O157:H7 *fliC* capture

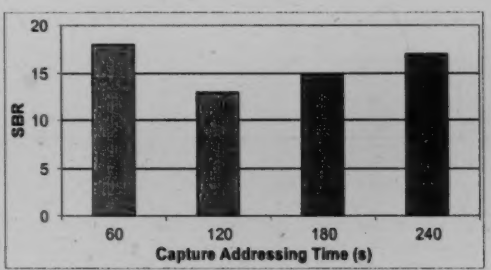


Figure 3.5 also demonstrates that lower SBRs were obtained with capture addressing times greater than 60 s. Increased addressing times may damage streptavidin bonds in the permeation layer, thereby decreasing the amount of available bonds for capture binding.

Sixty seconds was chosen as the capture addressing time for *V. cholerae* O1, *S.* Typhi and *E. coli* O157:H7 captures because it yielded the highest SBR with the shortest tested capture addressing time.

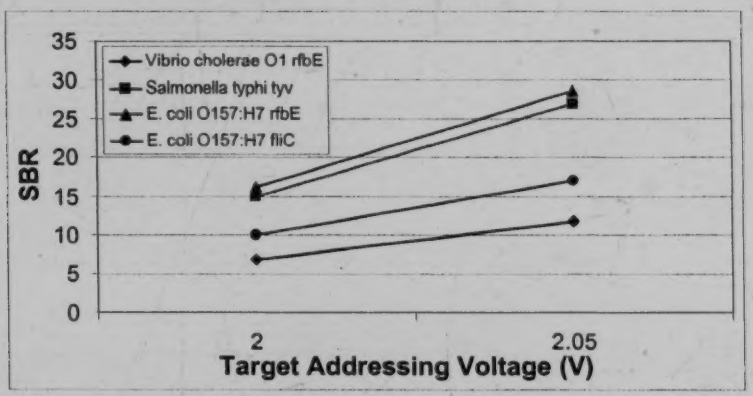
3.3.2.2 Target Addressing Voltage

Within the loader, target addressing voltage can also be adjusted from a minimum of 1 V to a maximum of 2.5 V. The default target addressing voltage is 2 V. It was necessary to evaluate this parameter because a low addressing voltage is desirable to reduce test site damage. As a result, the maximum voltage evaluated was 2.05 V.

Figure 3.6 demonstrates the results obtained following a 2 V and a 2.05 V target addressing voltage. At 2 V, the SBR values were 15:1 for *S.* Typhi *tyv*, 16:1 for *E. coli* O157:H7 *rfbE*, 10:1 for *E. coli* O157:H7 *fliC* and 7:1 for *V. cholerae* O1 *rfbE*. At 2.05 V, the SBRs from all four targets increased substantially: 27:1 for *S.* Typhi *tyv*, 29:1 for *E. coli* O157:H7 *rfbE*, 17:1 for *E. coli* O157:H7 *fliC* and 12:1 for *V. cholerae* O1 *rfbE*. Although the voltage was only increased by 0.05 V, SBRs almost doubled due to the increased positive bias on the activated test sites attracting significantly more negatively charged DNA molecules compared to 2 V.

A 2.05 V target addressing voltage was used because it yielded the highest SBR of the tested values for the *rfbE* and *fliC* gene of *E. coli* O157:H7, the *rfbE* gene of *V. cholerae* O1 and the *tyv* gene of *S.* Typhi without damaging the test sites.

Figure 3.6 Influence of target addressing voltage on SBR for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes



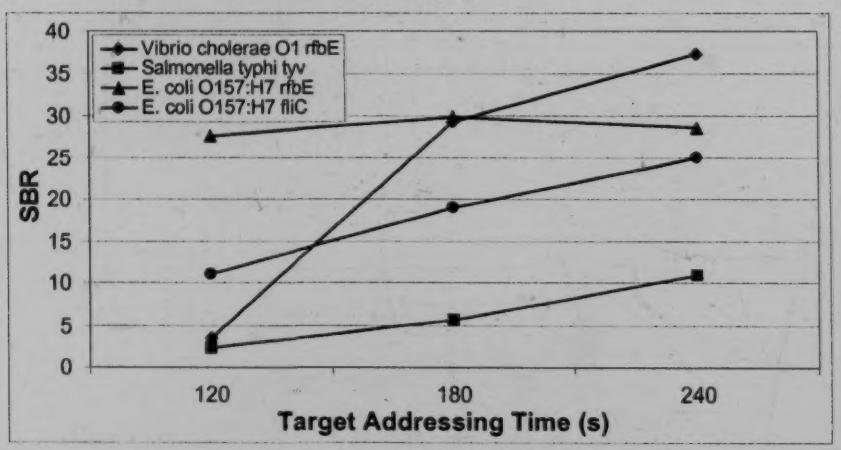
3.3.2.3 Target Addressing Time

Target addressing time can be adjusted from 10 s to a maximum of 600 s. The default target addressing time is 120 s. Three target addressing times were evaluated: 120, 180 and 240 s. Similarly to capture addressing time, target addressing time was investigated because of the different target lengths. Following RT-PCR, the target lengths were: *V. cholerae* O1 *rfbE* 419 bp, *S.* Typhi *tyv* 329 bp, *E. coli* O157:H7 *rfbE* 239 bp and *E. coli* O157:H7 *fliC* 179 bp.

As shown in Figure 3.7, following a 120 s target addressing time, *V. cholerae* O1 *rfbE* and *S.* Typhi *tyv* targets yielded low SBRs at 3:1 and 2:1, respectively, whereas *E. coli* O157:H7 *rfbE* and *E. coli* O157:H7 *fliC* SBRs were at 27:1 and 11:1, respectively. After 180 s, all targets yielded increased SBRs: 30:1 for *V. cholerae* O1 *rfbE*, 6:1 for *S.* Typhi *tyv*, 30:1 for *E. coli* O157:H7 *rfbE* and 19:1 for *E. coli* O157:H7 *fliC*. After 240 s, the SBRs were as follows: 37:1 for *V. cholerae* O1 *rfbE*, 11:1 for *S.* Typhi *tyv*, 28:1 for *E. coli* O157:H7 *rfbE* and 25:1 for *E. coli* O157:H7 *fliC*. Therefore, all target SBRs increased even more compared to 180 s, with the exception of *E. coli* O157:H7 *rfbE*.

Based on these data, an important trend can be observed. With increasing target addressing time, the SBRs increased substantially for longer targets. For example, the *V. cholerae* O1 *rfbE* SBR at 180 s increased 10 times compared to that obtained at 120 s. The *S.* Typhi *tyv* SBR at 180 s doubled compared to that obtained at 120 s. Based on these data, a 240 s target addressing time was used because it yielded the highest SBR for all targets among the tested values.

Figure 3.7 Influence of target addressing time on SBR for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes



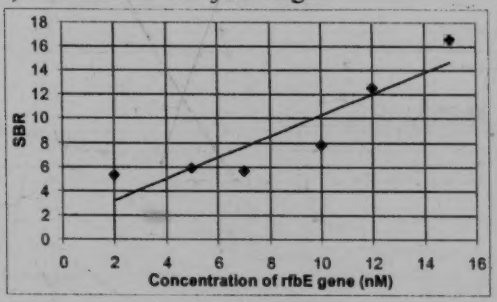
3.3.3 Target Concentration

The detection limit of the developed array technique was investigated using the following addressing parameters: a 60 s capture addressing time, a 2.05 V target addressing voltage and a 240 s target addressing time. Figure 3.8 shows the SBR obtained from target concentrations ranging from 1 nM to 20 nM. These data demonstrate that increasing SBRs are obtained with increasing target concentrations for all four targets. This trend is in agreement with previous work showing that the SBR is positively correlated with the target concentration [96, 123, 129].

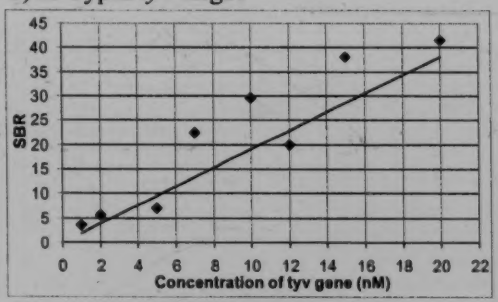
The DNA microarray technique detected low target concentrations of *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC*. As shown in Figure 3.8, it can clearly detect 1 nM target concentrations of *S.* Typhi and *E. coli* O157:H7. Also, at low target concentrations of 1 and 2 nM, the highest SBRs were obtained from the *rfbE* and *fliC* targets of *E. coli* O157:H7. At these two concentrations shorter targets yield higher SBRs because they are more easily addressed to test sites compared to longer ones.

Figure 3.8 Influence of target concentrations on SBR for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* genes

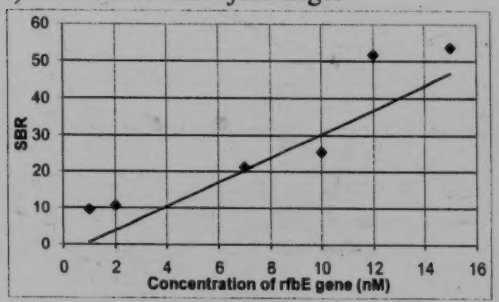
a) *V. cholerae* O1 *rfbE* target



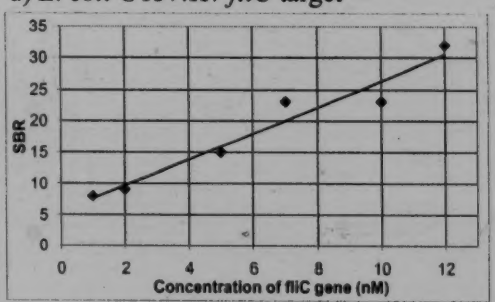
b) *S.* Typhi *tyv* target



c) *E. coli* O157:H7 *rfbE* target



d) *E. coli* O157:H7 *fliC* target



3.3.4 Detection Limit

The detection limit of the DNA microarray was compared to that of traditional methods such as agarose gel electrophoresis. 100 nM, 15 nM, 1 nM and 0.25 nM concentrations of *E. coli* O157:H7 *rfbE* and *fliC* DNA were separated and visualised by gel electrophoresis. As shown in Figure 3.9, *E. coli* O157:H7 *rfbE* and *fliC* DNA bands

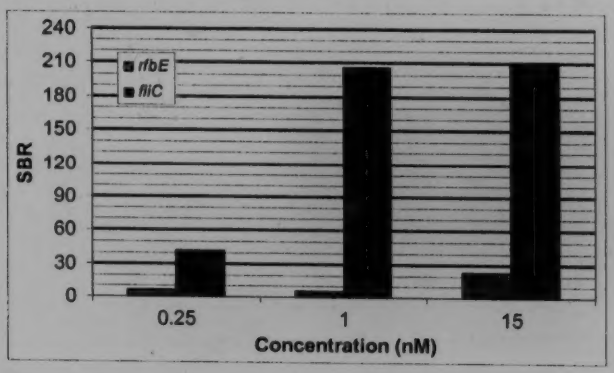
are visible in lanes corresponding to 100 nM and 15 nM *rfbE* (lanes 2, 3) and *fliC* DNA (Lanes 6, 7). However, below 15 nM, no DNA bands are visible on the gel (Lanes 4, 5, 8, 9).

Figure 3.9 Agarose gel electrophoresis of 100 nM, 15nM, 1 nM and 0.25 nM *E. coli* O157:H7 *rfbE* and *fliC* DNA

From the left:
Lane 1 – DNA ladder
Lane 2 – 100 nM *rfbE*
Lane 3 – 15 nM *rfbE*
Lane 4 – 1nM *rfbE*
Lane 5 – 0.25 nM *rfbE*
Lane 6 – 100 nM *fliC*
Lane 7 – 15 nM *fliC*
Lane 8 – 1 nM *fliC*
Lane 9 – 0.25 nM *fliC*

Three concentrations of *E. coli* O157:H7 *rfbE* and *fliC* DNA (15 nM, 1 nM and 0.25 nM) were evaluated using the optimised DNA microarray detection technique. As shown in Figure 3.10, SBRs of 7:1 for *rfbE* and 42:1 for *fliC* were obtained when the concentration was as low as 0.25 nM whereas 15 nM was required for agarose gel detection. Therefore, the DNA microarray was determined to provide a 60 times better detection limit compared to agarose gel electrophoresis.

Figure 3.10 SBR for *E. coli* O157:H7 *rfbE* and *fliC* DNA at 15 nM, 1 nM and 0.25 nM



As discussed in section 3.2.9, detection limit was also compared using three different *E. coli* O157:H7 cell numbers. RT-PCR was performed on total RNA that had been extracted from 150 cells. The DNA obtained following RT-PCR was then diluted 10 times to approximate 15 cells and 100 times to approximate 2 cells. Figure 3.11 shows the results obtained following agarose gel electrophoresis of these DNA samples. The *fliC* and *rfbE* DNA from 150 cells is visible on the gel (Lanes 2 and 5, respectively). In the 10 times dilution, a faint band was visible for *fliC* DNA (Lane 3) and therefore, a positive identification can be made whereas no DNA band was visible for *E. coli* O157:H7 *rfbE* DNA (Lane 6). In the 100 times dilution, no DNA bands were visible for *fliC* or *rfbE* (Lane 4 and 7, respectively). As a result, detection limit for the agarose gel was estimated to be approximately 15 cells for the *E. coli* O157:H7 *fliC* DNA and 30 cells for the *E. coli* O157:H7 *rfbE* DNA (*rfbE* data shown in Figure 2.7).

Figure 3.11 Agarose gel electrophoresis of *E. coli* O157:H7 *fliC* and *rfbE* DNA obtained from 150 cells, a 10 times dilution (approximately 15 cells) and a 100 times dilution (approximately 2 cells)

From the left:
Lane 1 –DNA ladder
Lane 2 – *fliC* DNA from 150 cells
Lane 3 – *fliC* DNA from a 10 times dilution
Lane 4 – *fliC* DNA from a 100 times dilution
Lane 5 – *rfbE* DNA from 150 cells
Lane 6 – *rfbE* DNA from a 10 times dilution
Lane 7 – *rfbE* DNA from a 100 times dilution

Next, the same concentrations of *E. coli* O157:H7 DNA was run on the microarray to determine the SBRs. Figure 3.12 demonstrates that SBRs of 6-8 were obtained from a 10 times dilution (approximately 15 cells) of *E. coli* O157:H7 DNA following array detection whereas at the 100 times dilution (approximately 2 cells), the SBR values were 2-3.

Figure 3.12 SBR data for *E. coli* O157:H7 *fliC* and *rfbE* DNA obtained from 150 cells, a 10 times dilution (approximately 15 cells) and a 100 times dilution (approximately 2 cells)

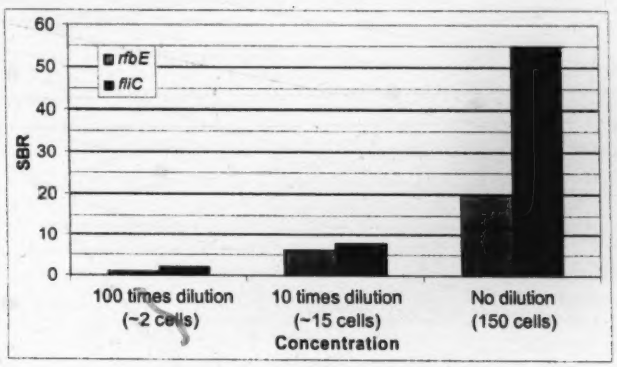
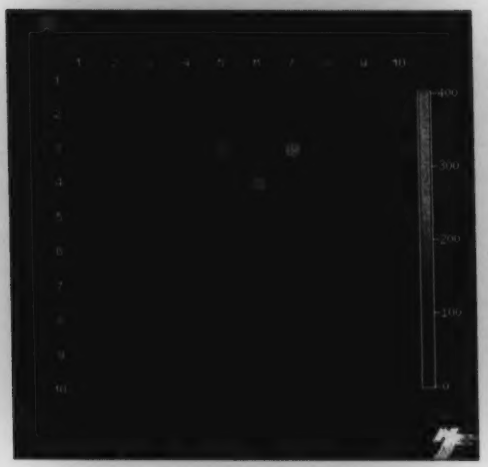


Figure 3.13 shows the same results displayed in the format obtained from the NanoChip™ configuration software. This figure shows all test sites on the array and their corresponding fluorescence intensity is displayed on the right hand side.

Figure 3.13 Fluorescent test sites on the array corresponding to *rfbE* and *fliC* DNA obtained from 150 cells, a 10 times dilution (approximately 15 cells) and a 100 times dilution (approximately 2 cells)



Sites are indicated according to their (X, Y) location
(5,1), (7,1), (6,2): negative control
(5,3), (7,3), (6,4): *fliC* DNA from 150 cells
(9,1), (8,2), (10,2): *rfbE* DNA from1 50 cells
(5,5),(7,5), (6,6): *fliC* DNA from a 10 times dilution
(9,3), (8,4),(10,4): *rfbE* DNA from a 10 times dilution
(5,7), (7,7), (6,8): *fliC* DNA from a 100 times dilution
(9,5), (8,6), (10,8): *rfbE* DNA from a 100 times dilution

As a result, the DNA microarray was determined to have a lower detection limit than agarose gel electrophoresis for *rfbE* DNA from *E. coli* O157:H7. For *fliC* DNA, the array was not an improvement because there was a DNA band visible on the gel in the 10 times dilution lane (approximately 15 cells) (Lane 3, Figure 3.11). However, this DNA band is hard to visualise. As discussed in section 1.8.2., even though DNA microarray analysis is more expensive compared with gel electrophoresis, it is a much quicker and more sensitive detection technique and, as such, can yield more accurate results for hard to visualize DNA bands.

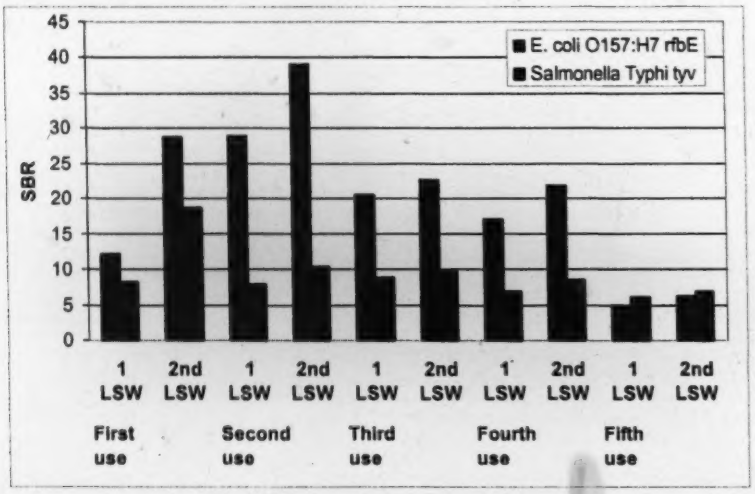
These results are in agreement with those of Chizhikov et al. [73], who also observed the improved detection limit of arrays compared to agarose gel electrophoresis.

They found that following PCR, *E. coli* O157:H7 *fliC* DNA was not visible on a gel; however, the product did produce a detectable signal on the array. Wilson et al. [62] also demonstrated a detection limit of 10 fg *Bacillus anthracis* DNA that was not detectable on an agarose gel.

3.3.5 Re-usability

To examine how many times *tyv* and *rfbE* capture loaded test sites could be repeatedly used, a cartridge was sequentially reloaded with *tyv* and *rfbE* target. After each repeated use, target was readdressed to the same capture loaded test sites and SBRs were obtained following two low salt washes (LSW). Figure 3.14 shows the SBRs following five repeated uses. From these data, two important trends can be identified. First, SBR always increased after the second low salt wash, demonstrating the efficiency of low salt washes in removing unbound reporter, thereby decreasing the background fluorescence. Second, for the *tyv* gene of *S.* Typhi, the SBR remains constant at around 10:1 until five repeated uses at, which point the SBR is close to 5:1. For the *rfbE* gene of *E. coli* O157:H7, beyond three repeated uses, the SBR remains close to 20:1 and similarly to the *tyv* gene, drops to 5:1 at five uses. These data are very promising because they indicate that a capture loaded cartridge can be reused up to five times with little effect on SBR.

Figure 3.14 SBR of 15 nM *E. coli* O157:H7 *rfbE* and *S.* Typhi *tyv* targets obtained from repeated use of the DNA microarray



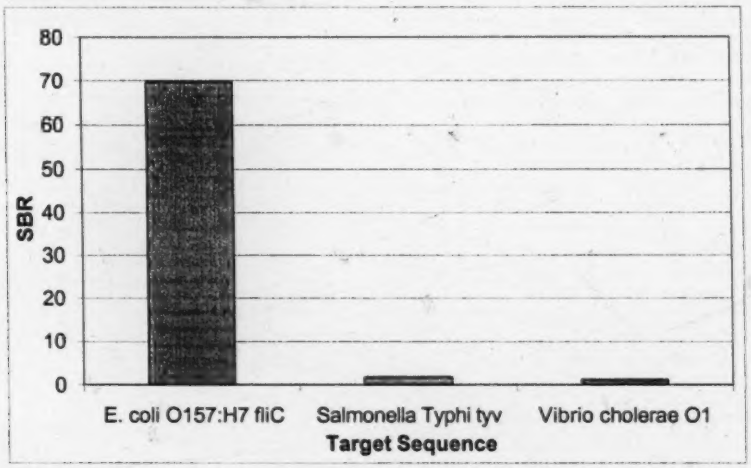
Note: there is an increase in SBR after the second use of the array for the *rfbE* gene of *E. coli* O157:H7. This increase may have resulted from an increased target and/or reporter hybridisation compared to the first run or an error in the preparation of that concentration.

3.3.6 Specificity

Specificity was evaluated by determining the SBR obtained with a complementary capture and target hybrid compared to that of a mismatch capture and target hybrid. *E. coli* O157:H7 *fliC, S.* Typhi *tyv* and *V. cholerae* O1 *rfbE* targets were addressed individually to *E. coli* O157:H7 *fliC* capture loaded sites. SBRs were obtained following the hybridisation of *E. coli* O157:H7 *fliC* reporter to the array. As shown in Figure 3.15, *E. coli* O157:H7 *fliC* capture loaded test sites addressed with complementary *E. coli* O157:H7 *fliC* target yielded a SBR of 70:1 whereas sites addressed with mismatch targets *S.* Typhi *tyv* and *V. cholerae* O1 *rfbE* yielded SBR values of 2:1. These data indicate that the *E. coli* O157:H7 *fliC* capture and reporter are specific for *E. coli*

O157:H7 *fliC* target and that only minimal hybridisation will occur with mismatch targets.

Figure 3.15 SBR obtained with 15 nM *E. coli* O157:H7 *fliC*, *S.* Typhi *tyv* and *V. cholerae* O1 *rfbE* targets addressed to *E. coli* O157:H7 *fliC* captures and reported by the *E. coli* O157:H7 *fliC* reporter



3.4 Conclusions

This chapter has documented the development of a DNA microarray technique for *V. cholerae* O1 *rfbE*, *S.* Typhi *tyv* and *E. coli* O157:H7 *rfbE* and *fliC* gene sequences. The addressing parameters chosen for these targets were a 60 s capture addressing time, a 2.05 V target addressing voltage and a 240 s target addressing time. A relationship of increasing SBR with increasing target concentrations was obtained for 1-20 nM concentrations.

The DNA microarray was determined to be provide a lower detection limit compared to agarose gel electrophoresis because it detected 0.25 nM concentrations of *rfbE* and *fliC* DNA as well as a 10 times dilution (approximately 15 cells) of *E. coli* O157:H7.

Initial experiments show that the DNA microarray can specifically detect *E. coli* O157:H7 *fliC* target. Future studies will build on this experiment and evaluate the specificity of the other three captures and reporters.

Chapter 4: Summary and Future Research

A reverse transcription single-tube MPCR assay was successfully developed for the amplification of viable *E. coli* O157:H7, *Salmonella* Typhi and *Vibrio cholerae* O1 using primers for the *E. coli* O157 lipopolysaccharide (LPS) gene (*rfbE*), the H7 flagellum gene (*fliC*), the *V. cholerae* O1 LPS gene (*rfbE*), and the *S.* Typhi LPS gene (*tyv*).

To ensure that the assay detected only viable replicating bacteria, total RNA from each bacterium was extracted and treated with DNase to remove any contaminating DNA and ensure that only RNA was detected. This was further confirmed by a dead cell control consisting of approximately 10^7 *E. coli* O157:H7 cells that had been boiled at 100°C for 10 min. No DNA bands from this dead cell control were detected using agarose gel electrophoresis, confirming that the dead cells did not contain *rfbE* and *fliC* mRNA. Therefore, the RT- MPCR assay specifically detected viable *E. coli* O157:H7.

The assay specificity was further validated using seven blinded RNA samples and two groups of bacteria obtained from clinical specimens. All seven blinded RNA samples were correctly identified to contain every combination of these three bacteria using agarose gel electrophoresis. Furthermore, the assay was used to detect clinical isolates and to determine whether other bacteria interfered with the detection and identification of the target *E. coli* O157:H7 and *S.* Typhi bacteria. Analysis of the group 1 bacteria containing non-pathogenic *E. coli*, *E. coli* O157:H7, *Listeria monocytogenes*, *Yersinia enterocolitica* and *S.* Typhi demonstrated that the assay specifically amplified the *rfbE* and *fliC* genes of *E. coli* O157:H7 and the *tyv* gene of *S.* Typhi. Following analysis of the group 2 bacteria without the target bacteria, no DNA bands were detected; therefore, no

false positive results were obtained from selected bacteria that did not contain the target mRNA.

The detection limit of the assay was determined using four different concentrations of template RNA: 100 nM, 50 nM, 20 nM and 5 nM. The RT-MPCR assay with agarose gel electrophoresis detection required 50 nM of total RNA to confirm the presence of all three bacteria. The assay detection limit was also evaluated using a series of dilutions of bacteria obtained from clinical specimens: 75000 cells, 7500 cells, 1500 cells, 150 cells and 30 cells. As few as 30 cells of *E. coli* O157:H7 and *S.* Typhi were positively detected on an agarose gel.

The second major contribution of this thesis was the development of a DNA microarray technique for detection of *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1 to improve the detection limit and sample throughput. The development process involved the successful design of gene-specific captures and reporters for each of the four bacteria gene sequences. Next, sample addressing parameters were investigated and parameters were chosen that yielded the highest SBR and the shortest addressing time of the tested values. These include a 60 s capture addressing time, a 240 s target addressing time and a 2.05 V target addressing voltage. The relationship between the target concentration and the SBR was evaluated to explore the potential of this assay for quantification. The SBR increased with increasing target concentrations for all four bacterial gene sequences.

The detection limit of the DNA microarray detection technique was evaluated using 15 nM, 1 nM, and 0.25 nM DNA. The DNA microarray technique was able to clearly detect 0.25 nM of DNA, whereas gel electrophoresis required 15 nM. The microarray detection provided a 60 times lower detection limit compared to agarose gel

electrophoresis. The detection limit of the microarray technique was further examined using different numbers of *E. coli* O157:H7 bacterial cells: 150 cells, a 10 times dilution (approximately 15 cells) and a 100 times dilution (approximately 2 cells). Detection of as few as 15 cells (from the 10 times dilution) was achieved; therefore, the DNA microarray was capable of detecting fewer cells.

The specificity of capture and reporters was evaluated following the hybridisation of 15 nM of *E. coli* O157:H7 *fliC*, *S.* Typhi *tyv* and *V. cholerae* O1 *rfbE* targets. Positive detection of the *E. coli* O157:H7 *fliC* gene was obtained without interference from the other targets, demonstrating the specificity of the capture and reporters for *E. coli* O157:H7 *fliC* gene.

To reduce the cost of the assay, the re-usability of the cartridge containing capture loaded test sites was investigated. A cartridge containing the captures for the *rfbE* gene of *E. coli* O157:H7 and the *tyv* gene of *S.* Typhi were repeatedly addressed with targets after the removal of the previous runs targets and reporters. A SBR higher than 5:1, the criterion for positive identification, was obtained in up to five uses. The ability to reuse the DNA microarray chip substantially reduces the analysis costs and makes this technology well suited to high-throughput applications.

This thesis has presented the development of an innovative and specific detection assay for viable pathogenic bacteria. Future research should be continued on this study in two areas: viability markers and further development of the DNA microarray technique.

Viability markers are urgently required for many applications, including monitoring drinking water supplies. In fact, it is essential to distinguish bacteria that are actively producing virulence factors from those that are dead. This is necessary to prevent

false positives that result in unnecessary boil water advisories. On the other hand, false negative results can have even more devastating consequences; thus, a reliable detection method for viable bacteria is crucial in protecting public health. Although mRNA holds promise as an accurate viability marker, a number of fundamental questions remain unanswered. For example, the degradation of the *rfbE* gene of *V. cholerae* O1, the *tyv* gene of *S.* Typhi, and the *rfbE* and *fliC* genes of *E. coli* O157:H7 mRNA under a variety of treatments including standard water disinfection processes such as chlorination, ozonation, high and low temperature treatments is unknown and needs to be investigated. Also, ethanol treatment, which theoretically destroys RNases should also be evaluated in order to test the hypothesis by Sheridan et al. [59] that slower mRNA degradation results from the destruction of RNases. Finally, the existence of mRNA in VBNC should be explored to determine if they are preserved in this dormant state.

Because the DNA microarray offers a lower detection limit, it should be further developed to allow for the simultaneous detection and differentiation of *E. coli* O157:H7, *S.* Typhi and *V. cholerae* O1. Also, as in real-time PCR, the targets should be fluorescently labelled during PCR amplification, thereby eliminating the need for reporter probes. This improvement will reduce analysis time and eliminate mismatch target/reporter hybrids.

Once the DNA microarray technique has been established, future studies will need to evaluate the potential interference from a wide variety of bacteria, including those normally found in water and clinical specimens.

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